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BACTERIOPHAGE TYPES OF *SHIGELLA SONNEI* IN QUEENSLAND

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In 1949, a major outbreak of dysentery caused by *Shigella sonnei* occurred in Brisbane, Queensland (Singer and Ludford, 1953). The first case to come to our notice was near a housing camp, and subsequently the infection appeared among occupants of the camp. It soon extended to other areas near the original focus of infection, and in three months had spread throughout the city area. Over two hundred cases of *Sh. sonnei* dysentery appeared in nine months. The organism had not been found in the period since 1947 when the Institute began investigations; earlier records are difficult to obtain in Brisbane, but no isolation could be discovered.

Hammarström (1949) described a method of classifying *Sh. sonnei* into a number of types by means of bacteriophages, using the rough (R) form, as it was found to give better results than the smooth. Moreover, this form was always readily obtained, while the smooth (S) form soon disappeared from old cultures. Eleven phages were used, and a large number of types could be differentiated by the varying patterns obtained on the strains of *Sh. sonnei* under examination. A twelfth phage was used as an indicator for roughness, giving a confluent lysis with almost all R forms of whatever type. By this method 31 types of *Sh. sonnei* were established, and a further 37 were isolated on a single occasion only. Although the number seems large, type stability *in vivo* was satisfactory, though not absolute. Probably 0·6 p.c. changes of type occurred. *In vitro* stability of types kept for some months in artificial culture was less, as 3·4 p.c. of strains were found to have changed. Similar results have been obtained in the present investigation.

No other usage of this typing had appeared at this time, and it was thought the Brisbane outbreak would be a suitable occasion to determine its value in Australia.

Sh. sonnei was first reported in Australia by Patterson and Williams (1922), six strains being found in Melbourne. Beare (1923) reported the organism in Adelaide, South Australia, and it was described as the cause of infantile diarrhoea by Webster and Williams (1925) in Melbourne. Before the war the organism was again reported in Melbourne on several occasions (Burnet, 1930; Nelson, 1932; Farren-Ridge and Lush, 1933). During the war it was reported by Rothstadt *et al.* (1943); two strains being isolated from dysentery cases

in an Australian General Hospital. Draper (1944) reported the organism in Adelaide, and noted that it caused hospital infections. It also appeared in the Northern Territory, where isolation of three strains was reported by Wall and Garvan (1945).

A recent outbreak in Sydney has been described by Williams (1951). The first cases appeared in a hospital ward in March, 1949, and were followed by more cases admitted from the city area. Although only 61 were seen, it was thought that the outbreak was much more widespread, as many were mild and not sent to hospital where a bacteriological examination could be made. Although *Sh. sonnei* has been reported frequently in Australia during the last twenty-five years, that by Williams is the only large outbreak described in the literature.

With this history of *Sh. sonnei* in mind, an attempt has been made by examining material from different Australian States to discover whether there has been any connection between outbreaks in recent years. However, it has not been possible to go far in this direction because of the difficulty in obtaining strains from epidemics which occurred more than a year or so ago.

Methods.

Lines of bacteriophage and stock type strains of *Sh. sonnei* were obtained from Dr. F. Hammarström, Sweden. The technique of typing was essentially the same as used by him (1949).

The preparation of bacteriophage was carried out by picking a single plaque, together with a little of the surrounding culture, into 15 ml. of heart infusion broth (Difco), and incubating at 37° C. for five hours. The preparation was then passed through a Horman Ekwip 3·6 mm. D7 Seitz type filter. This was always found sufficient to sterilize, although the finer D9 filter is equivalent to the Seitz EK sterilizing pad. The phage preparations were then checked for sterility.

The determination of the highest dilution of bacteriophage preparation giving confluent lysis of the test organism (C.T.D.) was performed as described by Hammarström and checked for the twelve phage preparations by means of his key (Hammarström, 1949, Table 18, page 63). The titre of stock preparations of bacteriophages was not found to alter appreciably over a year, and checking against the key was only performed when the original preparations were standardized. Type 8 was used as host strain of eight phages, in place of type 4 as used by Hammarström, who showed that the two strains gave identical results with the above eleven phages. C.T.D.'s of the phages were found to be: I, 1 in 30; II, 1 in 200; III, 1 in 8; IV, 1 in 500; V, 1 in 400; VI, 1 in 1,600; VII, 1 in 2,000; VIII, 1 in 1,600; IX, 1 in 400; X, 1 in 8; XI, 1 in 800; XII, 1 in 8,000.

It was shown by Hammarström that *Sh. sonnei* phages varied considerably in action when the composition of the medium was in any way changed. His original medium had to be modified to use materials locally available, but satisfactory results were obtained with the following formula, which was used during all work of typing and standardization of phage preparations.

Bacto proteose peptone	10·0 gm.
Lab. Lemco	5·0 gm.
Sodium chloride	3·0 gm.
Difco powdered agar	12·5 gm.
Distilled water to	1,000 ml.
pH 7·4	

Sterilize by autoclaving 15 lb. 15 minutes.

Typing technique was as follows: Standard platinum loops of 24 gauge wire were prepared by wrapping the wire closely about the shank of a No. 32 drill bit (diam. 2·95 mm.). Plates of the standard medium were inverted in the incubator at 37° C., and dried for 1 hour with lids removed. Loopfuls of broth culture were applied to the plate, and directed to give circular areas of 1–1·5 cm. in diameter. The plates were then carefully placed in the incubator for 30 minutes to dry with lids partially removed. When dry, phage was applied to the centre of each inoculated area. Care was taken, as advised by Craigie and Yen (1938), not to touch the loop on the inoculated area, but, by tilting the loop into a vertical plane, to allow its contents to run out. Twelve areas of culture were applied to each plate, and each was inoculated with a phage in regular sequence from I to XII, using phage XII to check that the organism under test was in the (R) phase. Tests were incubated at 37° C. for 5–6 hours, and then read against a fluorescent light.

Material Investigated.

Strains isolated by the Institute during the first three months of the outbreak of *Sh. sonnei* dysentery in Brisbane were kept in stab culture at room temperature. Following this period, no strains were kept until typing commenced eight months later. Material was also obtained from the Brisbane Children's Hospital, the Mater Misericordiae Children's Hospital, and from several Maternal and Child Welfare Homes about the city.

From the Brisbane Children's Hospital 85 strains were isolated in the first three months; of these only 64 were alive when typing commenced. In the corresponding period at the Mater Hospital, 23 strains were isolated of which 16 were typed.

For eight months, none of the isolated strains from the Brisbane Children's or the Mater Hospital was kept, 117 and 23 strains respectively being in this group.

When typing commenced, all strains isolated by the Institute from the Brisbane Children's and the Mater Hospitals were typed, together with some from the Pathology Laboratory of the Brisbane General Hospital. One hundred and eleven cultures were examined from the Brisbane Children's and 11 from the Mater Hospital in this period, which lasted ten months.

Five months after these investigations, it was decided to examine a number of additional cultures, in order to determine what had happened to types present when the last examinations ended. Nineteen strains make up this group.

Cultures from three Child Welfare Homes were examined at different periods of the investigation. From the Home S, at the beginning of the outbreak, seven of 37 cultures were typed. Home W was investigated by typing seven cultures from the 10 which were isolated in the indicated three-month period, and 10 cultures of the 34 isolated were typed from the remaining home.

A number of cultures were obtained from other laboratories throughout Australia. Fifty-five were examined from other States and two which had been isolated at Rockhampton, Queensland, in 1937.

RESULTS.

The number and reactions of the phage types found in this examination may be seen in Table 1. Also included are the reactions of three strains which did not fit any listed in the type scheme. A fourth reaction fits that of a provisional type 89 listed by Hammarström, and the last reaction is that given by two strains isolated at Rockhampton, Queensland, in 1937. This fits type 7 of the

antigenic scheme, but the colonial appearance of these strains differed from the usual (R) form of *Sh. sonnei*. The organisms appeared to have reverted to an "extra rough" form, probably from long storage on laboratory media. Too much reliance, then, should not be placed on the typing of these two strains.

TABLE 1.

Reactions of phage types found in this investigation.

Type	Source	Phages I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
3	N.S.W., Q'land	—											
5	N.S.W.	—	—										
12	Q'land, N.S.W.			—	—								
17	Q'land	—		—	—								
19	N.S.W., Vic., S. Aus., Q'land	—		—			—						
28	N.S.W.	—	few plaques		many plaques						—		
?	N.S.W.	—	±	—	—	—	—	±	—	—		±	—
?	2 strains B.G.H.	—	—	—		±	—	—		—	—		—
?89	1 strain B.G.H.	—		—			—	—		—			
?7	2 strains Rockhampton	—		—									

— = negative reaction. ± = semi-confluent lysis. No reaction marked = confluent lysis.

As there was no selection of patients entering the two children's hospitals, the results of typing from both have been combined in Table 2. Of strains isolated during the first two weeks of the outbreak, only the first was lost, so the first group of cultures examined well represents the beginning of the epidemic, and all proved to be phage type 19. In the two periods of typing to August, 1951, 185 of 200 strains tested belonged to phage type 19. Three strains could not be allotted to any position in the type scheme. The significance of the other types will be discussed later.

Cultures from Homes S and W, where *Sh. sonnei* appeared soon after its introduction to Brisbane, were all found to belong to type 19. Home N, which was free from dysentery until late 1951, was infected by a different organism, all cultures examined belonging to type 17.

Brisbane strains examined from all sources in this period totalled 224, of which 199 belonged to phage type 19.

Results of the final period are of great interest, and are also shown in Table 2. Of 17 strains examined, 10 belonged to type 17, showing that this organism, which had just appeared in the last typing period, had become well established, and was now responsible for a high percentage of dysentery cases. It was this type which was responsible for the cases of dysentery in Home N.

TABLE 2.

Number and phage types of Sh. sonnei *cultures isolated in Brisbane.*

Source	Period	Strains examined	Strains not examined	Phage type 3	12	17	19	?
Children's Hospitals	Nov. 22, 1949 to Feb. 23, 1950	78	28	1			76	1
	Feb. 24, 1950 to Oct. 31, 1950		140					
	Nov. 1, 1950 to Aug. 15, 1951	123			3	9	109	2
	Jan. 7, 1952 to Mar. 24, 1952	19	46			11	8	
Home S	Feb. 20, 1950 to Mar. 20, 1950	7	30				7	
Home W	Mar. 17, 1951 to June 18, 1951	7	3				7	
Home N	Oct. 1, 1951 to Mar. 17, 1952	10	24			10		
	Total	244	271	1	3	30	207	3

Phage types of the interstate strains appear in Table 3, and help in evaluating local findings. The largest number of strains belonged to type 19, which was present in Sydney, Melbourne and Adelaide. Type 3 organisms were demonstrated from Sydney, Melbourne and Hobart, where this type apparently caused a number of cases of dysentery before the introduction of type 19. Types 5 and 28 were found only in New South Wales.

TABLE 3.

Phage types of Sh. sonnei *strains isolated in other States.*

State	No. of strains	Date Isolated	3	5	12	17	19	28	?
N.S.W., Sydney	6	June–Dec., 1949	4					1	1
" "	7	Mar.–Apr., 1951					7		
" "	10	Apr.–May, 1951	1	1			2	6	
" Uranquinty	5	May, June, 1951		1			4		
Victoria, Melbourne	3	1946	3						
" "	3	May, 1950 to 1951					3		
Tasmania, Hobart	1	Sep., 1949	1						
South Australia, Adelaide	17	Apr.–May, 1951					17		
Western Australia, Perth	3	June, 1952			3				

DISCUSSION.

No strain of *Sh. sonnei* type 19, isolated prior to the Brisbane epidemic, has been found among strains from other Australian States. Thus, typing has not provided any direct evidence as to the source of the Brisbane epidemic, but it seems likely that the strain may have arrived with migrants from Europe. The sequence of events indicated that a single focus of infection on the southern side of the city was responsible for this outbreak, which rapidly became widespread.

The relationship with the Sydney outbreak described by Williams (1951) is not clear. Only five strains isolated in Sydney between the beginning of the Sydney epidemic and the beginning of the Brisbane one were examined. Four of these belonged to type 3 and one to type 28. The typing does not indicate that *Sh. sonnei* type 19 arrived in Brisbane from Sydney, but the amount of evidence upon which this conclusion rests is small. The Sydney epidemic, on the other hand, undoubtedly seems to have been a mixed one, as four different types were found among the 23 strains examined. It looks as if Sydney would have been a better field for *Sh. sonnei* epidemiology than Brisbane proved to be.

The single case in Brisbane of an infection due to a type 3 organism was not investigated, as the culture was not typed until some months after isolation. As type 3 had occurred in Sydney and Melbourne, it seems likely that the infection came from the south.

The cases of infection due to *Sh. sonnei* type 12 show well the value of phage typing. In January, 1951, a strain of *Sh. sonnei* was isolated from a child migrant living in a migration hostel. Examination showed it to be type 12, which had not been found in Brisbane. The child had been in Queensland for two months, so it seemed possible that infection had come from a carrier in the hostel. Two months later, a fatal case of Sonne dysentery occurred in a child from the same hostel, and the organism obtained at post-mortem was found to be type 12. A few days later, another strain of type 12 was detected, again in a child migrant. At this point a bacteriological examination of the migrant hostel seemed desirable, but permission could not be obtained. Fortunately, no more cases were reported, and no hospital cross-infection occurred. The organism has not been found in Brisbane since these three infections took place. Had it not been for the typing, it would inevitably have been thought that a local strain had extended into the camp, and not that a new strain had been introduced into Brisbane. It is felt that the discovery of these types in migrant children is significant, and reflects the difficulty of preventing the arrival in Australia of diseases in which symptomless carriers play an important part. Over a year later, three cultures received from Perth, Western Australia, proved to be type 12. The children were not migrants, but no other information could be obtained.

The isolation of type 17 came first from migrant children, but then the organism spread by cross-infection in the Children's Hospital. Home N possibly

became infected from this source, as a child from the Children's Hospital was admitted to Home N two weeks before the first cases appeared. Five months later, when the final batch of cultures were examined, *Sh. sonnei* type 17 was causing about 50 p.c. of the cases in Brisbane, and had appeared in every institution where investigations were made. It is interesting to compare the extent of spread of type 12, which caused only three known cases, and type 17, which became widespread throughout Brisbane. Although type 17 may have had more opportunity to spread, it seems possible that its success may have been due to a greater ability to cause cross-infection, in fact to having been a more efficient pathogen.

The success of type 19 is even more apparent, as many hundreds of cases of Sonne dysentery arose from probably only a single focus of infection. Three years after its introduction, strains of this type could still be isolated in Brisbane. That the type could be demonstrated over such a long time also indicates its stability. While this type could be followed continuously for three years, the introduction of other types during this period was readily demonstrated, and the arrival of each fitted the epidemiological history of the cases. With a new technique, such as this method of phage typing, it is important to determine whether such arrivals of different phage types can be explained. If they can, the hypothesis that the type is really a new introduction and not a change of phage type *in vivo* is strengthened.

Since this work has been finished, studies of stability of phage types have been published by Mayr-Harting (1952), who showed that change of type occurred in strains exposed to certain bacteriophages. For example, *Sh. sonnei* type 3, which was sensitive to all but one phage, gave rise, after exposure to phage III, to a number of resistant strains, all less sensitive to the twelve test phages. Possibly the queried organisms in Table 1 are of this nature, the three strains isolated from the Children's Hospital having arisen from type 19. None of these strains apparently spread or caused cross-infection, which would have suggested the introduction of a new type rather than a change of type. On the other hand, it seems unlikely that types 12 or 17 arose from the common type 19, as both have different phage patterns and are not resistant to a greater number of phages. Moreover, the epidemiological evidence fitted the introduction of a new infection in both cases.

Conclusion.

The examination of outbreaks of intestinal infection by bacteriophage typing is becoming well established abroad (e.g., Boyd, 1952), but has not been used widely in Australia, other than the investigations of *Salmonella typhi* by Tolhurst *et al.* (1945) and Buckle (1946). The observations reported here are a further example of its usefulness, and it might profitably be extended to the study of other widespread intestinal pathogens also.

SUMMARY.

An outbreak of *Shigella sonnei* dysentery in Brisbane, Queensland, was investigated by the bacteriophage typing technique of Hammarström.

Two hundred and seven of 244 cultures examined were found to belong to type 19, which was responsible for all except one of the infections examined in the first 12 months of the epidemic. Its origin was not determined.

Types 12 and 17 were later found in a migrant hostel, but only type 17 became established in Brisbane.

Types isolated in other Australian States proved to be 3, 5, 12, 19 and 28. Six cultures, two from Queensland and four from other States, could not be typed.

It was concluded that phage typing of *Sh. sonnei* is of considerable value, when correlated with other epidemiological evidence.

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THE ANTIBIOTICS OF *ESCHERICHIA COLI*

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In recent years attention has been drawn to the production of inhibitory substances by strains of *Escherichia coli*. This phenomenon was described by Nissle in 1916.

Gratia (1932) showed that one strain of *E. coli* produced a substance active against another strain of *E. coli* and less so against *Shigella shiga*. This substance was produced in broth cultures of the organism, and was described as heat resistant, being slowly destroyed at 120°C.; it was destroyed by alcohol, but not by acetone or quinine, and was diffusable. It was not self-reproducing, and this, with the other properties, distinguished it from a bacteriophage.

Gratia and Fredericq (1946), in a series of short papers, found the production of inhibiting compounds to be very common in the coliform group. Such substances, for which they proposed the name "colicines," were also active against certain Shigella and Salmonella strains. They postulated the existence of a number of colicines, the presence or absence of members in the mosaic being responsible for the antibiotic spectrum of the producing organism.

Fredericq and Levine (1947) described a considerable number of enteric organisms producing substances active against an equally diverse range of other enteric organisms. Most frequently attacked were the *Shigella* group.

Heatley and Florey (1947) worked with a strain of *E. coli* producing an antibiotic active against another *E. coli*. The substance was formed in aerobic conditions, and could be produced in broth only during vigorous aeration, or by growing the organism on the outside of a cellophane sac containing broth. It was also quite heat stable, and probably a polypeptide.

Fredericq (1948) has published an extensive review containing many references on the colicines. At least 17 different active principals were detected, which differed in range of activity, type of inhibition zone produced as a sensitive organism, and resistance to heat and proteolytic enzymes.

The antagonism by *E. coli* strains has recently been studied by Halbert and co-workers (Halbert, 1948a; Halbert and Magnuson, 1948; Halbert and Gravatt, 1949), and the relation of antibiotic producers to *Shigella* infections examined (Halbert, 1948b, 1948c). A strain of *Shigella paradysenteriae* Flexner type III was used as test organism for determining production of inhibitors. Like that described by Heatley and Florey, the inhibitory substances were formed

only under aerobic conditions, but these workers could not demonstrate formation in broth, even when it was vigorously aerated. Consequently, work was done on extracts from agar media, good yields of antibiotic being obtained from some strains. Substances were quite heat stable, precipitated by 40–60 p.c. saturated ammonium sulphate, and also by acetone. The molecules appeared to pass with difficulty through dialyzing and ultrafiltration membranes. Halbert and his co-workers also concluded that the active substances appeared to be of peptide nature, but that they showed slight differences in physical properties from those investigated by Heatley and Florey.

It seems evident that there are a number of active substances produced by strains of *E. coli*. Until recently there has been no one simple method of comparing their properties. In most investigations strains of *E. coli* had not been characterized, but, with the advent of serological classification introduced by Kauffmann (1947), it is now possible to do this reliably.

A method of electrophoresis inside an agar gel was used by Gordon *et al.* (1950) for separation of large molecules. It was thought to apply this technique in an attempt to separate active substances produced about an inoculum of active *E. coli* on a nutrient agar.

Materials and Methods.

Twenty-seven type strains of *E. coli* containing "O" antigens 1–25, 55 and 111 were tested for antibiotic-producing powers. These were kindly provided by Dr. F. Kauffmann, for the production of immune group sera. Strains of *E. coli* isolated from children in welfare homes were also tested for activity, which was determined by the method of Halbert (1948a), but a locally isolated strain of *Shigella sonnei* in the rough form was used as indicator. This organism belonged to phage type 19 as determined by the typing method of Hammarström (1949).

To test activity, 0·5 ml. of a 1:10,000 to 1:20,000 dilution of a 24-hour broth culture of *Sh. sonnei* was spread on a 10 cm. 0·1 p.c. glucose heart-infusion agar plate. After drying an hour at 37° C., *E. coli* strains were inoculated in small circular areas, and the plate incubated overnight at 37° C. Width of inhibition zone from the edge of the *E. coli* inoculum to the commencement of normal size *Sh. sonnei* colonies, was then estimated.

For electrophoretic examination, two strains were tested on each plate. Heart-infusion agar in 35 ml. amounts was poured into 15 cm. petri plates and allowed to harden. Excess agar was then excised with a sterile knife, to leave a strip 5 cm. wide across the diameter of the plate. The

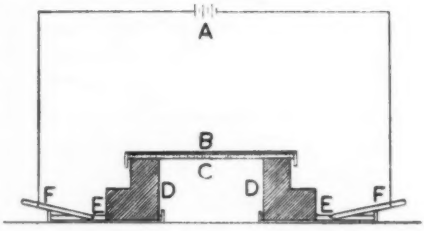

Fig. 1. Diagram of electrophoresis system used for the examination of *E. coli* strains.

A. 45 volt battery.
B. petri dish with the agar strip under test.
C. colony of *E. coli* under test.
D. agar blocks.
E. phosphate buffer in petri dishes.
F. carbon electrodes.

strains under test were then placed halfway along the strip in 2–3 mm. inocula, a few millimetres from the edge, and the plate incubated overnight at 37° C. The organisms were then killed by exposure for 30 minutes at 37° C. to chloroform placed in the lid of the plate. Excess chloroform was removed by inversion of the two parts of the plate in the incubator for fifteen minutes. The plate, with lid removed, was then inverted on two blocks of heart-infusion agar standing in petri dishes containing 10–15 ml. of Sorensen's phosphate buffer of pH 7·4. Carbon electrodes connected to a 45 volt "Radio B" battery were then placed in the petri dishes (Fig. 1).

To prevent overheating of the agar, a tin containing ice was placed on the plate containing the strip. With potentials higher than 45 v., this cooling device was inadequate, and cracking and dehydration of the agar occurred. Electrophoresis was usually carried out for five hours, as dehydration increased if this period was extended, and in a longer time the faster moving components possibly would have run over the strip.

After electrophoresis was concluded, the plate, with the lid removed, was inverted in the incubator for a few minutes to remove any condensed moisture. The agar strip was again exposed to chloroform vapour for approximately fifteen minutes in an attempt to prevent contamination. Excess chloroform was removed as described earlier.

Finally, 0·8 ml. of a 1 in 5,000 dilution of a 24-hour broth culture of the indicator *Sh. sonnei* strain was run on the strip and spread with a platinum wire. Results were read after incubation overnight at 37° C.

TABLE 1.

Kauffmann type strains of E. coli *and their activity against* Sh. sonnei.

Strain designation*	"O" group	Activity, inhibition zone in mm.	Strain designation	"O" group	Activity, inhibition zone in mm.
5	1	2 mm.	F7902	15	2·5 mm.
9	2	3 mm.	F11119	16	3 mm.
14	3	2·5 mm.	F12a	17	non
4	4	2·5 mm.	F10018	18	non
1	5	non	F8188	19	non
7458	6	non	P7a	20	non
7509	7	1 mm.	E19a	21	non
3404	8	non	E14a	22	2·5 mm.
316	9	1 mm.	E39a	23	non
8337	10	non	E41a	24	2 mm.
623	11	non	E47a	25	non
626	12	non	1064	55	non
4321	13	non	?	111	non
4411	14	non			

* These are Dr. Kauffmann's designation of the cultures received from him.
? = Strain designation not known.

RESULTS.

Table 1 shows the results obtained when the twenty-seven Kauffmann strains were tested against *Sh. sonnei*. Ten strains were found to produce an inhibition zone.

Table 2 shows the number of active and inactive strains in 26 serological groups of *E. coli*, which were isolated from 141 persons in Brisbane. Both the number of isolations and the number of persons from whom these isolations were made is shown in each case.

TABLE 2.

Number of locally isolated active and inactive strains, together with their serological groups.

"O" group	Number active		Number inactive		"O" group	Number active		Number inactive	
	Isolations	Persons	Isolations	Persons		Isolations	Persons	Isolations	Persons
1	21	8	14	9	14	—	—	—	—
2	18	6	21	14	15	7	2	5	2
3	—	—	24	11	16	—	—	2	1
4	5	2	64	33	17	—	—	9	3
5	—	—	2	2	18	2	2	23	17
6	48	9	45	18	19	—	—	3	2
7	—	—	41	15	20	4	1	—	—
8	2	2	15	9	21	5	4	71	22
9	2	1	2	1	22	—	—	—	—
10	—	—	28	6	23	—	—	1	1
11	—	—	4	2	24	—	—	—	—
12	13	8	3	2	25	4	2	18	6
13	—	—	2	2	111	7	5	86	29

There is some evidence that the production of inhibitors is not a permanent property and that strains which were previously negative may begin to produce them. Changes were observed in organisms belonging to group 4 and group 111, and are shown in Tables 3 and 4 respectively. The isolations were made from a group of children in the same welfare home. Most strains which belonged to these two groups were found not to produce inhibitors, as may be seen in Table 2. Table 3 shows the appearance in child A of a group 4 organism which was active against *Sh. sonnei*. It occurred in three successive examinations of the faeces, then two weeks later a similar organism appeared in the faeces of child B. Active organisms were also present in the next two examinations of this child, but inactive strains appeared in the third specimen. Two weeks later, inactive group 4 organisms were shown to be present in the faeces of both children.

Table 4 traces group 111 organisms which were active against *Sh. sonnei*. First appearing in child C, the organism appeared in child D one week later, and in children E and F two weeks later. All except D excreted an inactive organism in their faeces at a later date.

It may be seen that organisms both active and inactive against *Sh. sonnei* were found in 12 of the 26 serological groups. In ten groups no organisms were found which produced antagonistic substances against *Sh. sonnei*. No inactive strains were found in group 20. Many non-groupable strains produced inhibitory substances, but are not included here.

TABLE 3.

E. coli *group 4 showing variable activity against* Sh. sonnei.

Date	Child A	Child B
17/7	3 colonies < 1 mm.	no group 4
23/7	2 colonies < 1 mm.	no group 4
30/7	1 colony 2 mm.	no group 4
6/8	no group 4	no group 4
13/8	no group 4	1 colony 1 mm.
20/8	no group 4	2 colonies 2 mm.
27/8	no group 4	1 colony 1 mm. 2 colonies not active
3/9 to 24/9	no group 4	no group 4
2/10	1 colony not active	1 colony not active

Figures in millimetres denote size of inhibition zones.

TABLE 4.

E. coli *group 111 showing variable activity against* Sh. sonnei.

Date	Child C	Child D	Child E	Child F
6/8	1 < 1 mm.	no group 111	no group 111	
13/8	1 < 1 mm.	2 < 1 mm.	no group 111	
20/8	no group 111	no group 111	2 < 1 mm.	2 < 1 mm.
27/8	no group 111	no group 111		2 not active
3/9	no group 111	no group 111	2 not active	
17/9	2 not active		no group 111	

The active Kauffmann strains were tested electrophoretically, using Kauffmann's strain of *E. coli* "O" 4 for comparison in each case. This organism was used, as preliminary observations showed that it produced two components which were just resolvable after electrophoresis at a potential of 45 volts for five hours. Most of the strains tested produced more than one area of inhibition at pH 7·4, four distinct electrophoretic spectra being recognized.

Fig. 2 shows the electrophorograms of the comparator strain and the Kauffmann strain of group 16. Both have two adjacent spots travelling to the negative electrode. The same two spots were obtained with strains of groups 4 and 22.

Fig. 3 shows another type of electrophoretic spectrum in the strain belonging to "O" group 1. This has a constituent travelling to the anode, as well as a fast-travelling constituent towards the cathode, with a trail. The same spectrum was given by strains of "O" groups 7, 15 and 24.

The third type of electrophoretic pattern obtained is shown with the organism of "O" group 2 on Fig. 4. It has only one fast-moving spot travelling towards the cathode. This spectrum is also given by the strain belonging to "O" group 9.

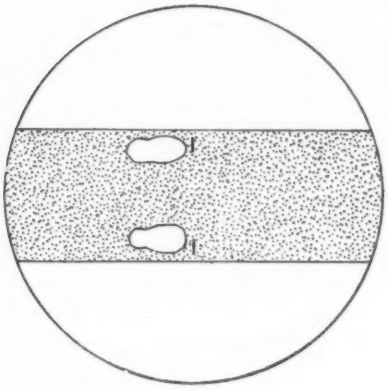

Fig. 2.

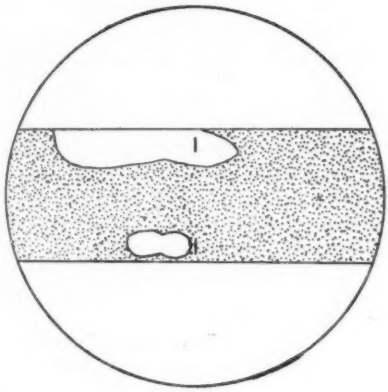

Fig. 3.

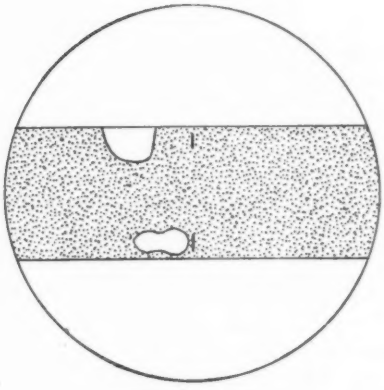

Fig. 4.

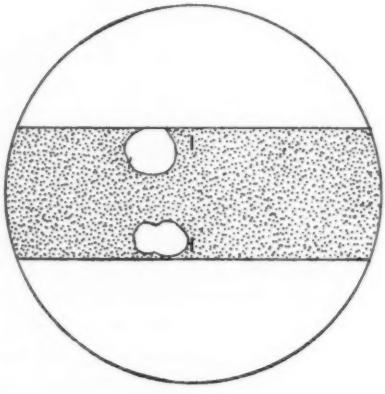

Fig. 5.

Fig. 2. Electrophoresis of comparator strain and *E. coli* group 16. Area of stippling denotes growth of *Sh. sonnei*, clear areas show inhibition spots.

Fig. 3. Electrophoresis of comparator strain and *E. coli* group 1.

Fig. 4. Electrophoresis of comparator strain and *E. coli* group 2.

Fig. 5. Electrophoresis of comparator strain and *E. coli* group 3.

The "O" group 3 strain appears to produce only the faster moving of the two components of the comparator strain and is the only example of this spectrum (Fig. 5).

Several inactive cultures, namely strains belonging to "O" groups 5 and 6, were also electrophorized and gave no areas of inhibition.

DISCUSSION.

It may be seen, from the examination of the Kauffmann type cultures and the locally isolated strains of *E. coli,* that serological grouping and antibiotic activity are independent of one another. In twelve groups, both active and inactive strains were demonstrated from Brisbane material. Active cultures were found in one more group in which no inactive members were isolated, while five strains of the Kauffmann collection were active, as opposed to the inactive strains of the same groups in the Brisbane cultures.

Although results from several hundred cultures are shown in Table 2, some "O" groups were rarely encountered or not at all, and it seems likely that, in a larger examination, strains would be found both active and inactive in all twenty-five groups. The incidence of Sonne dysentery has possibly an effect on the number of inhibitory *E. coli* strains in the population. This will be described in a further paper.

Some evidence has been found to indicate that strains may gain and lose their inhibitory powers, but full serological examination, including the "K" and "H" antigens, is necessary to follow this problem. Using the methods in this examination, it is impossible to follow anything but a slow change in a strain which has been observed for some time. Under these circumstances, it seems likely that any change would be an actual change in activity of the organism rather than a new strain introduced into the host.

Under certain conditions it was possible to use the technique of electrophoresis in an agar gel to compare inhibitory compounds produced by various strains of *E. coli.* It was found, however, that these conditions must be kept constant in each experiment, if plates are to be compared. For example, before the volume of agar used in pouring the plates was standardized, irregular results were obtained from day to day. Old batteries with variable charges were also a source of error.

It is evident, as found by Fredericq (1948), that a mosaic of antibiotics is produced by many strains of *E. coli.* Whether the presence of particular members of the mosaic is responsible for the antibacterial spectrum of active strains could readily be determined by this technique. It is possible that the technique described here may prove useful for isolating antibiotics and studying them individually.

SUMMARY.

Using the method of Halbert, 148 of 648 strains of *Escherichia coli* tested were found to produce substances inhibitory to *Shigella sonnei* type 19.

By using electrophoresis in strips of nutrient agar gel, it was found possible to distinguish four antibiotic patterns in the strains of *E. coli* tested. The production of antibiotics and their patterns was independent of the Kauffmann "O" group to which the organisms belonged.

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MUTATION OF DIFFERENT BACTERIOPHAGE TYPES OF STAPHYLOCOCCI TO STREPTOMYCIN RESISTANCE[1]

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(Accepted for publication 8th July, 1953.)

Investigations at the Royal Prince Alfred Hospital, Sydney, commenced in 1948, have shown that the majority of antibiotic-resistant strains of *Staphylococcus pyogenes* fall into distinct phage groups. There have been 1,357 isolates of types in the phage group 6/47 corresponding to Group III of Williams and Rippon (1952). Of these 49 p.c. were resistant to penicillin and 28 p.c. were resistant to both penicillin and streptomycin. By contrast, of the 301 isolates in the phage group 3A (Group II of Williams and Rippon), only 3 p.c. were resistant to penicillin and 0·6 p.c. resistant to both antibiotics. In the phage group 29/52 (Group I of Williams and Rippon) there were 246 isolates of which 8 p.c. were resistant to penicillin and 7 p.c. were resistant to both penicillin and streptomycin.

Barber and Whitehead (1949) observed that a high proportion of penicillin-resistant staphylococci isolated from patients in widely different hospitals belonged to the 6/47 phage group. The findings given above support these observations. These workers consider that it is possible that strains of the 6/47 phage group are genetically less stable than those of other phage groups and give rise to resistant mutants more readily.

Staphylococci which have become resistant to streptomycin *in vitro* appear to be identical with those which have become resistant *in vivo* (Barbour, 1950). This is not true of penicillin-resistant strains. It can be seen that resistance to streptomycin follows the same general pattern as resistance to penicillin. We have carried out the present investigation to determine if there are any differences in mutation rate to streptomycin among different phage groups of staphylococci.

METHODS.

Determination of mutation rate was carried out by inoculating 50 tubes containing 1 ml. of meat extract broth (pH 7·0) with between 50 and 200 viable cells. A meat extract agar plate containing 100 μg. streptomycin per ml. was inoculated at the same time with approxi-

[1] This work was commenced at the Fairfax Institute of Pathology under a grant from the National Health and Medical Research Council and completed at the N.S.W. University of Technology.

mately 1×10^4 cells from the same culture that was used to inoculate the tubes. This procedure was adopted to ensure that no resistant cells were used to inoculate the tubes. After incubation overnight 9 ml. of meat extract broth containing streptomycin were added to each tube to give a final concentration of streptomycin of 100 μg. per ml. of broth. A number of tubes to which no streptomycin had been added were set aside in an ice bath and subsequently used to estimate the average number of viable cells exposed to streptomycin. The method of counting was that described by Miles and Misra (1938).

The 50 cultures containing streptomycin were incubated for 72 hours and then examined for turbidity. Positive cultures were streaked onto both nutrient agar containing streptomycin at a concentration of 100 μg. per ml. and onto nutrient agar containing no streptomycin. Cultures which grew both with and without streptomycin were termed streptomycin resistant (SR) and those which grew only on the streptomycin agar were termed streptomycin dependent (SD).

Mutation rates were calculated from the formula of Newcombe (1948), which was modified from that of Luria and Delbruck (1943).

$$a = \frac{-(1n2)(1nPo)}{N}$$

where a = mutation rate per bacterium per division cycle.
Po = proportion of cultures having no resistant bacteria.
N = average number of viable bacteria exposed to streptomycin.

TABLE 1.

Mutation rates to streptomycin resistance.

Strain number	Phage group	Phage type	Mutation rate $\times 10^{-10}$	Mean rate for phage type $\times 10^{-10}$	Mean rate for phage group $\times 10^{-10}$
R1137	6/47	31B	1·27		
			0·60		
			0·70	0·86	
R1140	6/47	47/31B	0·97		
			0·88		
			0·80	0·88	
N794	6/47	7/47	0·97		
			1·19	1·06	
R1124	6/47	6/47	1·48		
			1·02		
			1·08		
			1·56		
			1·11	1·25	1·01
R1138	3A	3A/3B/3C	0·33		
			0·28		
			0·14	0·25	
R1147	3A	3B/51	0·15		
			0·17		
			0·18	0·16	0·21
R1153	29/52	29/31	0·39		
			0·50		
			0·57	0·49	0·49

RESULTS.

Four strains of phage group 6/47, two of group 3A and one of group 29/52 were tested for mutation rate to streptomycin resistance and streptomycin dependence.

Our main interest was in the SR mutants as these are of greater epidemiological significance. In the absence of streptomycin the SD types would die out. The results show that staphylococci of the 6/47 phage group mutate to streptomycin resistance approximately five times as rapidly as those in the 3A group and approximately twice as rapidly as the strain in the 29/52 group which we examined (Table 1).

Comparison of mutation rates to streptomycin dependence did not reveal such large differences between groups. The rate of the 6/47 group was approximately three times as high as that of the 3A group and approximately twice as high as that of the 29/52 group. The results are, however, more variable than those obtained for mutation rates to streptomycin resistance (Table 2).

TABLE 2.

Mutation rates to streptomycin dependence.

Strain number	Phage group	Phage type	Mutation rate $\times 10^{-10}$	Mean rate for phage type $\times 10^{-10}$	Mean rate for phage group $\times 10^{-10}$
R1137	6/47	31B	2·66		
			3·03		
			3·47	3·05	
R1140	6/47	47/31B	7·97		
			7·70		
			0·26	5·31	
N794	6/47	7/47	2·78		
			6·83	4·80	
R1124	6/47	6/47	1·48		
			1·24		
			7·75		
			7·53		
			4·20	4·44	4·40
R1138	3A	3A/3B/3C	3·49		
			2·55		
			1·37	2·47	
R1147	3A	3B/51	1·30		
			0·34		
			0·18	0·60	1·54
R1153	29/52	29/31	3·78		
			3·52		
			2·30	3·20	3·20

The streptomycin requirement of five SD isolates from strain R1124 was determined and found to vary from 1 to 100 μg. per ml. of broth. These results are set out in Table 3.

TABLE 3.

Streptomycin requirements of dependent isolates from strain R1124.

Isolate number	Streptomycin concentration—μg. per ml. of broth								
	0	0·1	1·0	5	10	15	30	60	100
SD1	—	—	—	—	+	+	+	+	+
SD2	—	—	—	—	+	+	+	+	+
SD3	—	—	+	+	+	+	+	+	+
SD4	—	—	—	—	—	—	—	—	+
SD5	—	—	—	—	—	—	—	—	+
Control	+	+	—	—	—	—	—	—	—

DISCUSSION.

Dobzhansky (1951) states that mutation rates in some strains of *Drosophila* are higher than in other strains of the same species, this high mutability being a genetically conditioned property of the strain. This worker believes that genetic variants which enhance or depress mutability are fairly common not only in *Drosophila* but in other organisms as well. Most of these mutability modifiers influence the general mutability rather than specific genes. Our findings support Dobzhansky's conclusions.

Our results support the suggestion of Barber and Whitehead (1949) that staphylococci of the 6/47 phage group are genetically less stable than those of other groups. This fact would account, at least in part, for the isolation in hospitals of a high proportion of streptomycin-resistant staphylococci belonging to this group. If the general mutability of this group has been enhanced it might be expected that the mutation rate to penicillin resistance would be higher than in other groups.

It will be interesting to note whether or not incidence of resistance to the newer antibiotics follows the same pattern as resistance to penicillin and streptomycin.

SUMMARY.

Comparison of mutation rates to streptomycin resistance of different phage groups of pathogenic staphylococci reveals marked differences between these groups. These differences may account for the high proportion of antibiotic-resistant strains isolated which belong to the highly mutable 6/47 phage group. Mutation rates to streptomycin dependence do not show such large differences between groups.

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THE PROSTHETIC GROUP OF LACTOPEROXIDASE

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(Accepted for publication 10th July, 1953.)

Whereas the identity of the prosthetic group of the known plant peroxidases has been firmly established as protohaem, the nature of the haem groups of the animal peroxidases is not yet understood. Foulkes, Lemberg and Purdom (1951) have shown that myeloperoxidase (of leucocytes) has certain properties similar to those of choleglobin. The work of Yakashuji (1939) and Theorell and Åkeson (1943) has shown, as was pointed out by the latter authors, that lactoperoxidase is not derived from myeloperoxidase and has a prosthetic group different from other known peroxidases.

The present paper describes further attempts to elucidate the nature of the haem group of lactoperoxidase.

EXPERIMENTAL.

The preparation of lactoperoxidase.

The enzyme was prepared from whole milk by a method to be published elsewhere. Each preparation was from approx. 70 l. milk handled in two to four lots over a ten-day period. The products from this first stage of purification (stored at —10° C.) were combined and purified to the level of 40–50 p.c. purity. The yield of approx. 50 p.c. pure enzyme is low; about 200 mg. of enzyme (in terms of pure enzyme) were obtained from 70 l. milk. This amount of enzyme would contain of the order of 1·3 mg. of lactoperoxidase porphyrin (assuming a porphyrin molecular weight of 600 per enzyme molecule and using the molecular weight of 92,700 found by Theorell and Pedersen (1944).

Attempts to split lactoperoxidase.

All attempts to split the enzyme into its protein and prosthetic group moieties have been unsuccessful. Theorell is also reported (Chance, 1950) not to have achieved this separation.

The following methods of separation have been applied. (1) Acetone–HCl treatment. This procedure, with variation of acid concentrations and of temperature, always precipitated the entire green haemoprotein. (2) Enzymic

[1]This work was supported by a grant from the National Health and Medical Research Council of Australia.

digestion followed by acetone–HCl treatment. Digestion with pepsin (pH 2·5, overnight at 37° C.) followed by trypsin (pH 8·5, overnight at 37° C.) gave, after adjustment of pH to 4·5 and addition of acetone, a fine green sludge quite insoluble in acetone. (3) The silver sulphate method of Paul (1950) used by him to break the linkage between porphyrin and cysteine in porphyrin *c*. When this method was applied to cytochrome *c*, in dilute acetic acid, free haematin was detected in the acid acetone extract by observing spectrophotometrically the shift in the position of the Soret band. With lactoperoxidase no haematin compound was detectable in the acetone extract. (4) Treatment with concentrated hydrochloric acid, sulphuric acid, hydrobromic acid or with strong sodium hydroxide did not yield any detectable haemin or porphyrin soluble in organic solvents.

The absence of a formyl group.

The positions of the haemochrome (haemochromogen) bands of lactoperoxidase at 565 mμ and 530 mμ (Theorell and Åkeson, 1943; Morell, 1953) do not suggest the presence of a formyl group in the enzyme porphyrin since all the haemochromes of the known formyl haem compounds have α bands in the region 580–586 mμ. Additional evidence of the lack of a formyl group in the enzyme haem was provided by the negative result of the test used by Lemberg and Falk (1951). These authors found that preparation of the haemochrome in the presence of hydroxylamine hydrochloride caused a considerable band shift towards the shorter wavelengths when the haem compound contained a formyl group.

Preparation of a porphyrin compound from lactoperoxidase.

Since a method of splitting off the enzyme haem group was not found, some information concerning the nature of this haem was sought by preparing a porphyrin compound directly from the haemoprotein. This was prepared as follows: The enzyme was precipitated from aqueous solution by the addition of cold acetone. After washing with acetone in the centrifuge it was dried under vacuum and dissolved with heating in glacial acetic acid with the minimal addition of pyridine. Approximately 0·2 ml. of a hot solution of ferrous acetate was then added to the haem solution under CO_2 and at 100° C. After 1 minute sufficient concentrated hydrochloric acid was added to give an acid reaction to Congo red. The strong bands of the acid porphyrin could then be seen using a hand spectroscope. The green precipitate obtained by the addition of acetone to the enzyme-digested lactoperoxidase was also treated by the ferrous acetate method to give a porphyrin compound.

The neutral porphyrin spectra were obtained from several preparations of undigested lactoperoxidase by neutralizing the acid porphyrin solutions with pyridine (neutral to Congo red). The positions of the absorption bands of these porphyrins are given in Table 1.

TABLE 1.

Comparison of porphyrin compounds from lactoperoxidase with monoacetyl deuteroporphyrin.

Porphyrin	Positions of absorption bands — mμ		
	Acid porphyrin (1)		Neutral porphyrin(2)
Porphyrin compound from lactoperoxidase (undigested)	615·6 (3a)	564·9 (3b)	630–633, 576, 552, 510–512, 412
Porphyrin compound from enzyme-digested lactoperoxidase	610·9 (4a)	561·7 (4b)	
Monoacetyl deutero-porphyrin	606·6	558·6	635, 577, 547, 508, 410

(1) Solvent mainly acetic acid + pyridine + approx. 3 p.c. HCl as obtained in the process of removal of iron from lactoperoxidase. Solvent for monoacetyl deuteroporphyrin similar. Band positions were measured in a Hartridge Reversion Spectroscope.

(2) Solvent for porphyrin compound from lactoperoxidase as for (1) neutralized to Congo red with pyridine + equal volume pyridine. Solvent for monoacetyl deuteroporphyrin equal volumes acetic acid and pyridine. Band positions were measured in the Hilger "Uvispek" spectrophotometer.

(3) Means for seven porphyrin compound preparations. Standard deviations of means = 1·55 (3a) and 1·06 (3b).

(4) Means for three porphyrin compound preparations. Standard deviations of means = 1·00 (4a) and 0·74 (4b).

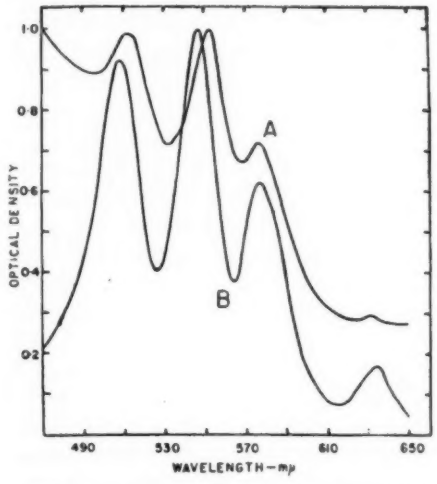

Fig. 1. The neutral absorption spectra of the porphyrin compound from lactoperoxidase (curve A) and monoacetyl deuteroporphyrin (curve B). The optical densities of both porphyrin solutions have been graphed so that the optical densities of bands III = 1·0. The measurements were made in a Hilger "Uvispek" spectrophotometer.

Unsuccessful attempts were made to bring the neutral porphyrin compound into solution in organic solvents such as ether, chloroform or amyl alcohol. It is assumed that this insolubility in organic solvents is due largely to a combination of the porphyrin with a non-porphyrin fragment (probably polypeptide).

The absorption spectrum of the neutral porphyrin compound is given in Fig. 1 together with that of monoacetyl deuteroporphyrin. This porphyrin was supplied by Dr. R. Lemberg, who separated it by chromatography on alumina from a mixture of the monoacetyl- and diacetyl-deuteroporphyrins.

DISCUSSION.

Because all attempts to split off the haem group of lactoperoxidase

have failed it has not been possible to study the free porphyrin derived from the enzyme. Nevertheless, the spectral properties of the porphyrin compound derived from lactoperoxidase give some evidence of the nature of the prosthetic group of the enzyme. The position and relative strengths of the absorption bands of the neutral and acid porphyrin compound have been compared with those of various known porphyrins. The closest correlation found was with monoacetyl deuteroporphyrin, the acid and neutral bands of which have been included in Table 1. The agreement between the positions of these two porphyrins is not perfect. Both porphyrins have a "rhodo" type spectrum in that the intensity of band III (552 mμ) is greater than that of band IV (513 mμ, see Fig. 1; the numbering is from the red end of the spectrum). There is close agreement in the positions of bands I and II of the neutral spectra but bands III and IV differ in the respective porphyrins by 4–5 mμ. In the acid spectra the lactoperoxidase porphyrin compound shows both bands approximately 5 mμ further towards the longer wavelengths.

In Fig. 1 the absorption spectrum of the porphyrin compound from lactoperoxidase shows considerable absorption in the troughs between maxima, compared to other porphyrins, particularly towards the blue end of the spectrum. Because of the impurity of the porphyrin compound the relative intensities of absorption of the various bands could not be compared on a quantitative basis. The ratio of absorption of band III to that of band IV for monoacetyl deuteroporphyrin was found to be 1·085 whereas that for the impure porphyrin compound from lactoperoxidase was 1·015 (Fig. 1). The increase in "trough" absorption at the shorter wavelength suggests that the ratio for the pure porphyrin compound may be greater than that found for the impure compound.

From Table 1 it can be seen that the bands of the acid porphyrin from enzyme-digested lactoperoxidase lie at wavelengths shifted approximately 3–4 mμ towards the blue end of the spectrum compared to those from the undigested enzyme. The bands of the acid porphyrin from the digested enzyme agree more closely with those of the acid monoacetyl deuteroporphyrin. This shift in absorption bands due to proteolytic digestion may be due to the saturation of a group such as a vinyl group by a protein fragment ($—CH = CH_2 \rightarrow —CHR—CH_3$). Such a band shift occurs in the conversion of protoporphyrin to haematoporphyrin. However, the above evidence plus, perhaps, the fact that bands III and IV of the neutral porphyrin compound are shifted 4–5 mμ towards the red end of the spectrum compared to bands III and IV of neutral monoacetyl deuteroporphyrin, is the only evidence of such an unsaturated side chain in lactoperoxidase porphyrin.

Because the free lactoperoxidase porphyrin has not yet been prepared and in view of the incompleteness of the data on the porphyrin-protein compound, it is not yet possible to establish the identity of the porphyrin. The comparison of the impure porphyrin-protein compound with monoacetyl deuteroporphyrin indicates that the porphyrin contains an acetyl side chain.

SUMMARY.

Unsuccessful attempts to split lactoperoxidase into its protein and haem moieties have been described.

A porphyrin compound, insoluble in organic solvents, has been prepared from the unsplit enzyme and its spectral properties examined.

The spectral similarity of the porphyrin compound from lactoperoxidase to monoacetyl deuteroporphyrin has been discussed. This comparison indicates that the porphyrin contains an acetyl side chain.

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THE PHARMACOLOGY OF SOME NEW ANTI-CHOLINESTERASES

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(Accepted for publication 16th June, 1953.)

Some time ago we reported a series of compounds with clear-cut antagonistic action towards morphine (Shaw and Bentley, 1952, 1953). Some of these substances were also potent anti-cholinesterases. This paper describes the unusual effects of substances which possess anti-cholinesterase activity but do not behave as does eserine on certain isolated organs, indeed on some occasions behaving as does atropine.

The previous work on choline esterase (C.E.) has been reviewed by Augustinson (1948). The most powerful antiesterase known is diisopropylfluorophosphonate, which gives 50 p.c. inhibition at a concentration of $1 \cdot 3 \times 10^{-9}$; eserine, the commonest, gives 50 p.c. inhibition at a concentration of 4×10^{-8}.

METHODS.

Choline esterase activity was determined by the manometric method of Ammon (1933). The effect on the isolated organs was investigated by standard pharmacological techniques which do not require further description. The drugs were added to the bath and the response of the organ was recorded. The effect of the drug on the response to ach. was then measured, whether potentiation or abolition (atropine action).

RESULTS.

The activity of the various substances tested is given in Table 1.

DISCUSSION.

These results make evident three paradoxes: (a) substances with and without anti-cholinesterase activity potentiate the action of ach. on certain organs, (b) the degree of potentiation varies greatly (from $\times 40$ to $\times 1$) with different organs, (c) some substances with marked anti-cholinesterase activity abolish the action of ach. on the heart, i.e. they have an atropine-like action.

As has been shown in a previous paper (Shaw and Bentley, 1953) there is no connection between anti-cholinesterase activity and morphine antagonism. In this paper the atropine effect of these compounds is again independent of the

TABLE 1.

Substance	Partition coefficient	Conc. giving 50 p.c. inhibition of C.E.	pKa	Guinea-pig uterus	Frog rectus	Isolated intestine (rat, rabbit, G.P.)	Straub heart (frog, toad)	Morphine antagonism	Remarks
1-Amino-acridine	1200	0	4·2	10^{-6} ach. × 20 r.w.o.	10^{-6} ach. × 1	2 × 10^{-6} ach. × 10 n.r.w.o.	2 × 10^{-5} stop heart 1 × 10^{-6} ach. × 1		No antiesterase activity but potentiates ach. in uterus and gut. No atropine action.
2-Amino-acridine	5·2	10^{-6}	8·2	10^{-6} ach. × 40 r.w.o. Tissue stained yellow. Spontaneous contractions.	10^{-5} ach. × 3 Tissue stained yellow. Sl. r.w.o.	2 × 10^{-6} ach. × 3 sl. contractions	2 × 10^{-5} ach. × 0	++	An antiesterase. Potentiates uterus markedly but rectus and intestine only slightly. Abolished action of ach. on heart (atropine action).
3-Amino-acridine	90	10^{-4}	5·8	10^{-6} ach. × 1 Spontaneous contractions.	2 × 10^{-5} ach. × 3 r.w.o.	2 × 10^{-6} ach. × 5 r.w.o. sl. conractions.	2 × 10^{-5} ach. × 0		An antiesterase. No potentiation on uterus, slight potentiation on rectus and intestine. Atropine action on heart.
4-Amino-acridine	55	10^{-5}	10·2	10^{-6} ach. × 10 r.w.o.	10^{-6} ach. × 2 r.w.o.	2 × 10^{-6} ach. × 1	2 × 10^{-5} no effect ach. × 1		An antiesterase. Potentiates uterus, slight potentiation on rectus, none on gut, no atropine action.
5-Amino-acridine	1·2	10^{-6}	9·9	10^{-6} ach. × 20 s. r.w.o. Higher concentrations paralyse muscle	10^{-5} ach. × 2	10^{-6} ach. × 10 strong contractions	10^{-4} no effect ach. × 1	+++	An antiesterase. Potentiates uterus and intestine but rectus only slightly. No atropine action.
1-Methyl-5-amino-acridine		10^{-6}	10·2	10^{-6} ach. × 10 r.w.o.	10^{-5} ach. × 2 sl. r.w.o.	10^{-6} no effect	2 × 10^{-5} ach. × 0 atropine action lasted for more than 1 hr.	++	An antiesterase. Potentiates uterus, rectus slightly, no action on gut. Marked atropine action.
Proflavine (2: 8-di-amino-acridine)	0·7	0	10·0	10^{6} ach. × 40	2 × 10^{-6} ach. × 2 sl. r.w.o.	2 × 10^{-6} slight transient contractions. ach. × 1.	2 × 10^{-5} ach. × 0 atropine action lasted for more than 1 hr.	0	No esterase activity. Marked potentiation of uterus, no potentiation of rectus or gut. Marked atropine action.

TABLE 1 (continued).

Substance	Partition coefficient	Conc. giving 50 p.c. inhibition of C.E.	pKa	Guinea-pig uterus	Frog rectus	Isolated intestine (rat, rabbit, G.P.)	Straub heart (frog, toad)	Morphine Antagonism	Remarks
Supraflavine (1: 9-dimethyl-2: 8-diamino-acridine)	0		8·7	10^{-6} ach. × 10 r.w.o.	10^{-5} ach. × 1				No esterase activity, potentiation of uterus but not of rectus.
Tetrahydro-monacrin (1,2,3,4-tetra-hydro-5-amino-acridine)	10^{-7}	9·9	10^{-6} ach. × 10 r.w.o. Spontaneous contractions.	10^{-5} ach. × 10	10^{-6} ach. × 10	10^{-4} slightly toxic. ach. × 0.	+++	Powerful antiesterase. Moderate potentiation of uterus, rectus and gut but atropine action on heart.
2-Amino-pyridine	0				2×10^{-5} no effect.	2×10^{-6} no effect	10^{-4} increased amplitude ach. × 0	0	No esterase activity. No potentiation of rectus or gut. Atropine action on heart.
4-Amino-pyridine	0				2×10^{-6} ach. × 0·8	10^{-5} spontaneous contractions ach. × 10	10^{-5} increased amplitude ach. × 0		No esterase activity. Potentiation of gut but not rectus. Atropine action on heart.
2: 6-Diamino pyridine	0				2×10^{-6} ach. × 1	10^{-5} ach. × 1	2×10^{-5} stop heart 1×10^{-6} ach. × 1	+	No esterase activity, and does not affect response to ach. on any tissue.
Phenyl diamino-thiazole (2: 4-diamino-5-phenyl-thiazole)	0			2×10^{-6} ach. × 1	2×10^{-6} ach. × 2·5 sl. r.w.o.	2×10^{-5} slight spontaneous activity ach. × 2	2×10^{-5} ach. × 0	+++	No esterase activity. Slight potentiation on rectus and gut but not on uterus. Atropine action on heart.

ach. × 20 etc. = potentiation of each 20-fold. r.w.o. = effect immediately reversible on washing out. sl. r.w.o. = reversible on washing out over 1 hour. n.r.w.o. = not reversible on washing out. ach. × 0 = effect of ach. abolished (atropine action).

enzyme factor. Albert *et al.* (1945) were able to show that bacteriostatic activity of the acridine series required complete ionization of the compound at pH 7·3—our results again show no such dependence. 3-Aminoacridine, which is 3 p.c. ionized at pH 7·3, potentiates the action of ach. on the frog's rectus and abolishes the action of ach. on the heart; likewise 2-aminoacridine, which is 88 p.c. ionized. Nor can the results be correlated with lipoid solubility. 1-Aminoacridine, with a partition coefficient of 1,200, potentiates the action of ach. on the uterus to the same degree as proflavine (partition coefficient 0·7). Substances with low partition coefficients may or may not show atropine activity.

As these physical-chemical properties do not provide an adequate explanation for the paradoxes, others (e.g. charge distance and ability to combine with the anionic and esteratic sites of C.E. (Nachmansohn, 1952)) require investigation.

As our results show that action is not commensurate with degree of ionization and lipoid solubility (two factors pre-eminently concerned with passage of substances through the cell membrane) it is possible that the various cellular effects considered by us took place at the cell surface. In particular, the potentiation or abolition of ach. action as a surface phenomenon would agree well with the findings of Clark (1933), and would fit in well with a cell receptor theory.

The action of the drugs in potentiating ach. may not reside entirely in their anti-C.E. activity, but may be due simply to an increase in excitability of the tissue, as a concentration of $1:10^6$ 5-aminoacridine caused a 10-fold increase of the response of the uterus to ergotoxin, and at a concentration of 4×10^6, a 6-fold increase in the response to pitressin.

SUMMARY.

The anti-cholinesterase activity of certain acridine, pyridine and thiazole derivatives has been measured. Some of these compounds are as active as eserine.

Some of these compounds potentiate the effect of ach. on certain tissues, while having an atropine-like action on others.

Acknowledgments. We wish to thank the National Health and Medical Research Council for a grant. Part of the expenses were also defrayed by the Bioassay Fund of the Physiology Department, to which the main contributors are Nicholas Pty. Ltd., Sigma Co. Ltd., and Burroughs Wellcome & Co. (Aust.) Ltd.

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OBSERVATIONS ON THE pH OF YEAST CELLS

by K. T. H. FARRER

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The importance of the pH of the cell contents of yeast can scarcely be overestimated and a number of workers have made attempts to measure it. Tait and Fletcher (1926), Small (1929), Mahdihassan (1930), Pfeiffer (1930) and Gutstein (1933) have investigated the pH inside the cell by various means, mainly colorimetric. It was generally agreed that the value is 5·9 to 6·3. However, a straight-forward electrometric study had not been made until Drews (1936) prepared expressed yeast juice, the pH of which he measured in two ways: (1) by the ordinary hydrogen electrode, i.e. in a stream of hydrogen; (2) with a U-electrode using a stationary hydrogen bubble. He obtained values of the same order and referred to the high buffering capacity of yeasts.

More recently, Gottschalk (1943) has compared the pH value of yeast liquefied by freezing and thawing, and by boiling with an equal volume of water. Using the glass electrode, he obtained virtually the same results, pH 5·9, for a bakers' yeast by each method, and confirmed by tests on the liquid centrifuged from the thawed yeast, that the considerable buffering power of yeasts is due to the salts. Gottschalk (1949) has again referred to the buffer system of the yeast cells in connexion with the influence of pH on the fermentation of glucose.

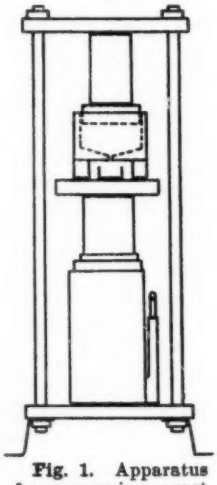

Fig. 1. Apparatus for expressing yeast juice.

The work reported in this paper was carried out in 1946 during the course of some studies on the proteolytic enzymes of yeast. It includes observations on the comparison of methods for determining the pH of yeast cells, the effect of the pH of the external medium on the pH of the yeast juice, subsequently expressed, and the measurement of the buffering capacity of yeast juice.

Methods.

The method of preparing the yeast juice was essentially that of Buchner 1903). The filter cell and sand were acid-treated and washed with water until a neutral reaction was obtained with B.D.H. Universal Indicator. The apparatus used was based on that of Lane and Hammer (1935) for the expression of cheese serum. The press consisted of three parts: cell, hydraulic jack and framework (Fig. 1).

pH was determined on successive 20 ml. aliquots of yeast juice as they came from the cell. The standard hydrogen and quinhydrone electrodes were unsatisfactory, the former because of severe frothing, and the latter because of the protein effect. The bubble type of hydrogen electrode was too cumbersome, consequently the glass electrode (Coleman) was adopted. This electrode does become poisoned with prolonged use, but this disadvantage was overcome by periodically washing the electrode in chromic-sulphuric acid solution followed by thorough rinsing with distilled water.

RESULTS.

The pH of yeast.

The pH of a number of samples of yeast was determined in three ways.

(i) On the liquid obtained by freezing and thawing the samples three times using a mixture of solid CO_2 and alcohol.

(ii) On the liquid obtained by boiling yeast with an equal volume of water.

(iii) On the expressed yeast juice.

The first two methods of treatment are those used by Gottschalk (1943). The second had been used extensively by Windisch, Kohlbach and Rothenbach (1928).

The results are set out in Table 1.

TABLE 1.

pH of yeast determined by three methods.

Yeast	Frozen and thawed	Boiled	Expressed juice
Bakers' Yeast I	5·74	6·22	5·92
" " II	5·53	5·53	5·71
" " III*	5·55	5·81	5·78
Brewers' Yeast I	5·87	6·32	5·95
" " II	5·83	6·26	5·83

* Bakers' yeast grown especially as a raw material for the manufacture of yeast extracts.

These figures show a lack of agreement between the three methods. In only two cases was the same result obtained by two methods. In two other cases agreement between two methods was very close. For each sample two separate aliquots (20 ml.) of yeast juice were measured, and two separate tests with boiling water were made. The second 20 ml. of yeast juice sometimes gave a slightly different pH value from the first (q.v.), but the second was taken as the correct one as the first 20 ml. was regarded partly as a wash for the press. Each time both boiling tests gave the same result. As the expressed yeast juice is composed of the actual contents of the macerated yeast cells, the values obtained for this were taken as correct. No auto-fermentation took place and the cells were shown by microscopic examination to be broken, whereas the cells were definitely unbroken by freezing or by boiling. The pH of yeast juice did not change over long periods, e.g. 24 hours at room temperature.

Juice from nine samples of yeast was collected in three successive 20 ml. portions and pH determined on each. The range of pH over the three samples varied from 0·05 to 0·23, with an average of 0·10. The pH of other samples of both brewers' and bakers' yeasts was determined only on the second 20 ml. of juice as has been described.

The results obtained have shown clearly that the pH values of all brewers' yeasts fall between 5·6 and 6·0 and that most of those studied are below 5·8. These figures are rather lower than the 6·0–6·3 suggested by the earlier workers but the same as that suggested by Drews (1936), pH 5·7, for similar yeasts. The bakers' yeasts showed some rather lower pH values. However, other results obtained have been outside the range of this series. Thus bakers' yeasts have been found with pH values as low as 4·6, and both bakers' and brewers' yeasts with values greater than 6 have been encountered.

The effect of the pH of the external medium on the pH of the cell contents.

As already indicated, more than one worker has referred to the difficulty of shifting the pH of the cell contents by changing the pH of the external medium. On the other hand, some (e.g. Dernby, 1917; Windisch *et al.*, 1928; Konovalov, 1938) have spoken of the optimum pH for the autolysis of yeast, which, initially at least, is an endocellular phenomenon.

30 gm. of brewers' yeast were mixed with N/10 solutions of NaOH and HCl, as indicated, and 350 ml. of water, and incubated at 52° C. for 30 minutes. The pH was measured at room temperature and the suspension filtered on a Buchner funnel. The yeast was washed very thoroughly to remove residual traces of the acid or alkaline medium and the cake sucked "dry." Yeast juice was then prepared. It was opalescent and coagulated on heating. In all respects it appeared to be true yeast juice. 20 ml. aliquots were collected and the pH determined on each separately. The results are given in Table 2.

TABLE 2.

pH of yeast juice prepared from yeast held at 52° C. for 30 minutes in adjusted media.

Volume of N/10 NaOH	400 ml.	100 ml.	Nil
„ „ „ HCl	100 „	400 „	„
pH of yeast suspension	7·0	4·6	—
„ „ „ juice	6·60	5·30	5·95

In each case there is a shift of approximately 0·6 pH units. This is quite appreciable. However, drastic steps were required to effect it, and there is evidence of the strong buffering power of yeast in the pH values of the yeast suspension, when the acid or alkali alone in the same volume would give pH valves of approximately 1·5 and 12·5 respectively.

The buffering capacity of yeast.

A measure of the buffering capacity of yeast is found in the pH titration curves, Figs. 2 and 3. To obtain that shown in Fig. 2, 42·6 gm. of brewers' yeast cake (equivalent to 10 gm. of yeast solids) were mixed with 25 ml. of N/10 HCl, allowed to come to equilibrium, and the pH measured. To the suspension was then added N/10 NaOH in the quantities shown, and the pH measured with the glass electrode when equilibrium was reached after each addition.

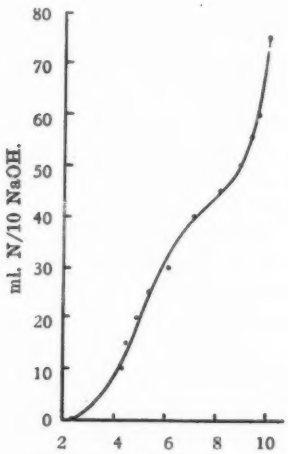

Fig. 2. Buffer curve for 10 gm. of brewers' yeast solids + 25 ml. of N/10 HCl titrated with N/10 NaOH.

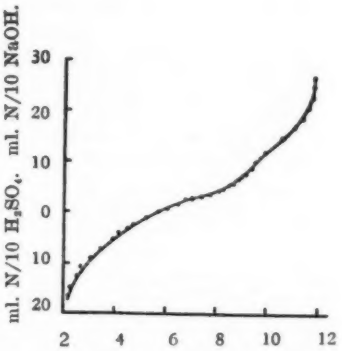

Fig. 3. Titration of brewers' yeast juice.

For Fig. 3, 5 ml. of yeast juice expressed from brewers' yeast were titrated with N/10 acid and N/10 alkali. The curve is very similar to that in Fig. 2 but less steep because of the small quantity of yeast solids represented.

The shape of both curves is strongly reminiscent of similar curves obtained with H_3PO_4, but the points of inflexion do not coincide. It would therefore be unwise to make too rigid a comparison even though it is known that phosphates must play a big part in the buffering of yeast.

DISCUSSION.

Drews (1936) obtained pH values of 5·66 to 5·73 with a normal hydrogen electrode, and, with the same yeasts, 5·82 to 6·16 with a bubble type electrode. He took the difference between the two electrodes to be a measure of the CO_2 effect.

From the results set down, it would appear that the most satisfactory method of determining the pH of yeast is a measurement by means of a glass electrode on the juice expressed from the macerated cells.

Provided no auto-fermentation or other disturbance of the cell contents had taken place, and there is no evidence of this, such a measurement is most likely to give the correct result.

Tait and Fletcher (1926), using colorimetric methods, had attempted to determine whether the reaction of the solution surrounding the yeast cell has any influence on the pH of the cell contents. They concluded that it had not. Drews *(loc. cit.)* confirmed this. He further pointed out that the pH of the cell contents is not a constant, but varies with the physiological state of the yeast. Tait and Fletcher had already implied this when, thinking of the work of Dernby (1917) on the various pH optima for the proteolytic enzymes of yeast, they suggested that autolysis was aided by a lowering of pH by acid production *inside* the cell. The difficulty of bringing this about by external influences was emphasized by Drews, who stated that "the reaction of the liquid surrounding the yeast cells has no effect on the pH inside the yeast cell. It is plain that the buffer mixture existing in the cell—carbonate, bicarbonate, primary and secondary phosphates, etc.—act as regulators." Gottschalk (1949) agrees with this.

It is clear from the results obtained that any attempt to control the endocellular processes of yeast by endeavouring to control the pH of the cell contents is unlikely to succeed because of the high buffering capacity of the cell. Such drastic steps are required as are likely, *per se*, to introduce other factors altogether.

SUMMARY.

The pH of yeast has been measured with the glass electrode on the expressed yeast juice. Comparisons were made with values obtained by measurement after boiling the yeast in water, or after freezing and thawing. The results obtained on the expressed juice are thought to be the correct ones. Brewers' yeasts gave values between pH 5·6 and 6·0. Most were below 5·8. Bakers' yeasts were somewhat lower.

The buffering capacity of yeast is clearly evident from the inability to shift the pH more than about 0·6 pH units with N/10 acid or alkali. Buffer curves for yeast suspension and yeast juice were obtained, and show some resemblance to that for H_3PO_4.

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A MECHANICAL CELL DISINTEGRATOR

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Disintegration of micro-organisms may be achieved in many ways (Werkman and Wood, 1940): rapid cooling, grinding (Booth and Green, 1940; Utter and Werkman, 1942; Wiggert *et al.*, 1940) supersonics, by means of a press (Hughes, 1952), or mechanical shaking with abrasives (Mickle, 1948).

For the study of intracellular particles, methods involving freezing are unsuitable, since freezing largely destroys the enzymic organization of such particles, e.g. mitochondrial "cyclophorase" (Harman, 1950). Ultra-rapid shaking with abrasives has proved most effective for extensive destruction of more resistant cells, such as yeast (Curran and Evans, 1942; Foulkes, 1951; Mickle, 1948; Northcote and Thorne, 1952).

This work describes a simple mechanical shaker, in which 2 gm. wet weight yeast or 0·5 gm. bacteria can be completely disintegrated in a few minutes, and some properties of the extracts obtained.

Methods.

The apparatus. Full plans for the shaker are given in Fig. 1. Its principle is simply the stepping up of the revolutions of a ½ h.p. motor by a factor of 4, giving about 5,600 cycles/minute. The ball races should be inspected after 10 hours' shaking and replaced if necessary. The various anchor bolts should be inspected frequently to see whether they have loosened.

Materials. The only abrasive used was Ballotini glass beads (The English Glass Co., Leicester, England), sizes No. 12 and 14. The three micro-organisms tested were *Saccharomyces cerevisiae* (as baker's yeast), *Proteus vulgaris*, grown on heart-infusion agar (Trudinger, 1951) and *Lactobacillus arabinosus*, grown on a complex medium (Nossal, 1951a).

Disintegration procedure. 2 gm. wet weight baker's yeast or *ca.* 0·5 gm. bacteria, 10 gm. Ballotini beads and 10 ml. 0·9 p.c. KCl were cooled to about 0° C. (without freezing) in the stainless steel capsule and shaken for not longer than 2 minutes at a time in a cold room at —1° C.; after 30 seconds disintegration, the temperature of the capsular contents was below 10° C., after 90 seconds below 20° C. The glass beads were not broken by shaking, could be recovered, washed and used again. After allowing the glass to settle out for a minute or so, the bulk of the liquid was decanted, examined microscopically and centrifuged for 10 minutes at 1,000 × *g* to remove glass, cell wall fragments and residual intact cells; the supernatant was used for dry weight measurements and assays of enzymatic activities.

Estimation of the extent of disintegration. Wet counts of unstained whole yeast cells and ghosts (empty cell walls) were made with an improved Neuberger haemacytometer on 1: 100 dilutions of the capsular contents after disintegration. The percentage disintegration $= 100 - \frac{\text{No. of intact cells}}{\text{No. of intact cells + ghosts}} \times 100$ (from the mean of five counted fields). Dry weights were measured at 120° C.

Enzymatic assays. Dehydrogenase activity was measured in standard Thunberg equipment, fumarase activity as the rate of malate formation from fumarate (Nossal, 1951b) and aspartase activity by ammonia formation from aspartate. Q values refer to μl./mg. dry weight/hour.

RESULTS.

Rate of disintegration and its reproducibility.

Table 1 shows the relation between shaking time, percentage disintegration and dry weight of the cell-free extracts for yeast cells. The reproducibility of disintegration for a given shaking period was within ±5 p.c. For shaking

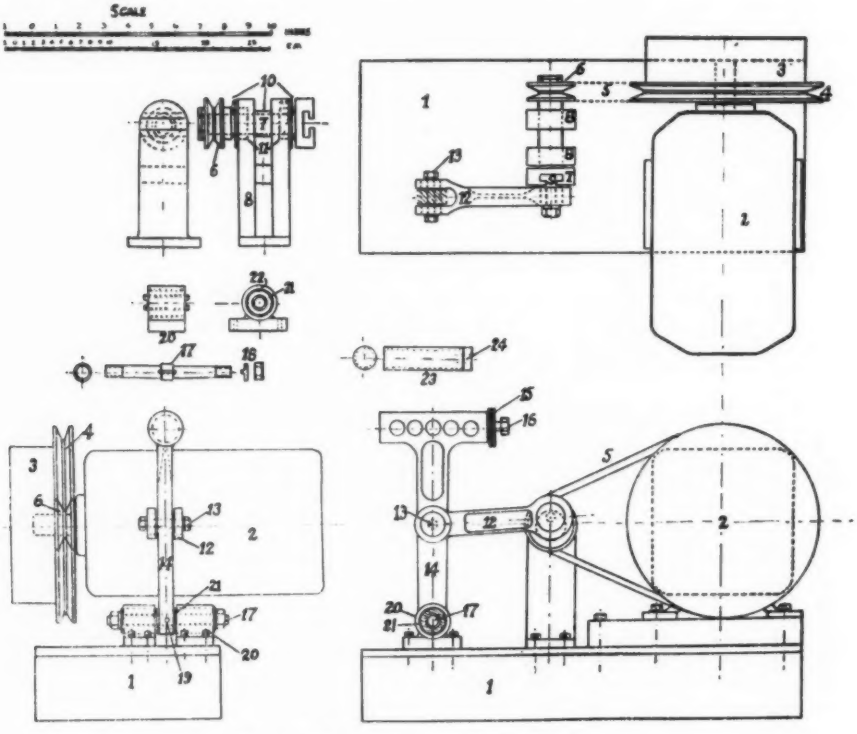

Fig. 1. Working Plans for the Shaker.

periods longer than 90 seconds, the accuracy of counting was lower because few intact cells remained and the ghosts were also largely disintegrated. For bacteria, rates of disintegration were done only semi-quantitatively: at an amplitude of 5/8″, up to 0·5 gm. wet weight bacteria required 2–3 minutes for complete disintegration, and larger amounts correspondingly longer. Fig. 2 shows yeast and *Proteus* preparations before and after disintegration.

TABLE 1.

Rate and reproducibility of disintegration of baker's yeast at an amplitude of 5/8″.

Shaking period (sec.)	No. of tests	Percentage disintegration		Dry weight of cell-free extract (mg./ml.)	
		Limits	Mean	Limits	Mean
10	5	26–38	33	12–17	14
30	20	47–55	53	20–24	21
90	10	79–89	82	27–33	30
270*	7	97–100	98	31–36	34

* Three 90-second disintegrations with 15 minutes cooling between the second and third.

EXPLANATION OF FIG. 1.

Main drawings:

(1) Base: fabricated mild steel and channel construction.
(2) Motor: ½ h.p. single phase, totally enclosed and moisture proof, A.C., 1,440 r.p.m.
(3) Clutch: Twiflex model C53, centrifugal, self-engaging at 800 r.p.m.
(4) Large pulley, cast aluminium, A section, V groove, 8″ p.c.d.
(5) Belt: A section, V belt, endless.
(6) As (4), 2″ p.c.d.
(7) Crank-shaft: H.T. steel, 2″ dia. reduced to 17 mm.
(8) Crank-shaft support: fabricated mild steel.
(9) Adjustable crank pin: H.T. steel, 1¼″ dia., reduced to ⅜″ on crank, sides flattened to stop rotation in slot and threaded for nut.
(10) Spacers: mild steel.
(11) 17 mm. ball-races, single row (SKF No. 6203).
(12) Conrod: cast duralium, containing at single end one 10 mm. S.A. ball-race (SKF 1200), at double end two 8 mm. double-row S.A. ball-races (SKF No. 108).
(13) Shaft: H.T. steel, 8 mm. dia., screwed both ends.
(14) Shaker arm: duralium 1⅛″ × ⅝″, with capsule holder welded on.
(15) Threaded capsule holder cap: duralium 1¼″ dia.
(16) Lock screw: 5/16″ S.A.E. brass screw.
(17) Pivot pin: H.T. steel, ¾″ dia., length 5½″, small dia. 7/16″ turned to fit rubber bearing, large dia. 0·627″, ⅝″ long, to fit shaker arm with 0·002″ interference, with two 3/32″ sq. keys, length 1⅜″, mild steel.
(18) Two keyed friction washers ⅛″ × ½″, with 3/32″ raised keys.
(19) No. 1 tapered pin.
(20) Bearing housing: fabricated mild steel, 1½″ dia. body, 2¼″ × 1⅝″ × ⅜″ base.
(21) Rubber bearings: Silentblock No. E.2070, slotted 5/32″ sq. to fit (17), pressed into (20) with 0·002″ interference.
(22) 1/16″ Allen grub screw, engages with C.S.K. in (20).
(23) Capsule: stainless steel, O. dia. 0·930″, I. dia. 0·870″, cap thread dia. ¾″ × 20 T.P.I.; length, external 3¼″, internal 3 1/16″.
(24) Cap: stainless steel, O. dia. 0·930″ × ¼″, knurled head, thread dia. ¾″ × 20 T.P.I.; length ½″, bored ½″ dia. to ⅜″ depth.

Enzymatic activities of the extracts.

Cell-free extracts of *Proteus* had Q values of 8,000 and 2,000 respectively for fumarase and aspartase. Both activities were found exclusively in the slightly opalescent supernatant obtained after 30 minutes centrifugation of the extracts at 3,800 $\times$ *g*. Here the Q values were 30,000 and 5,000 respectively.

In *Lactobacillus* extracts, the enzymes for the decarboxylation of pyruvate and acetolactate were again found in the supernatant fraction. Cocarboxylase and $MnCl_2$ were necessary for maximal pyruvate decarboxylation, $MnCl_2$ only for acetolactate. The Q_{CO_2} values were about 80 for pyruvate and 170 for acetolactate, compared with 80 and 140 respectively in the intact cell.

In yeast, the maximum $Q_{fumarase}$ activity of whole cell-free extracts was 750; the supernatant obtained after 15 minutes centrifugation at 3,800 $\times$ *g* gave Q values up to 1,500, showing that the enzyme predominated in the non-sedimentable fraction.

The extracts had no respiration in Warburg vessels at pH 7 and 38° C. in spite of the addition of metals and co-factors. However, the dialysed extracts showed the following decreasing order of dehydrogenase activities in Thunberg tubes (decolourization of methylene blue): ethanol, malic, lactic, succinic, citric, α-ketoglutaric, fumaric and glutamic. The following did not cause acceleration above the blank: pyruvate, acetate, oxaloacetate, glucose and acetaldehyde.

The succinic, α-ketoglutaric and lactic dehydrogenases were coenzyme-independent. After extensive dialysis, much malic dehydrogenase activity remained without addition of cozymase, suggesting either that there was some bound cozymase, or that a malic enzyme not requiring cozymase was also present. The ethanol dehydrogenase required cozymase. The citric enzyme was most active with added cozymase, $MnCl_2$ and adenosine-5-phosphate (Kornberg and Pricer, 1951).

When the extracts were fractionated by 30 minutes centrifugation at 3,800 $\times$ *g*, the succinic and α-ketoglutaric dehydrogenases were associated mainly with the residue, and the other enzymes showed higher activities in the supernatant. However, significant amounts of these "soluble" dehydrogenases were found even in twice-washed residues.

DISCUSSION.

The new shaker is simpler and more efficient than commercial models (Curran and Evans, 1942; Mickle, 1948). Northcote and Thorne (1952) report 30 minutes shaking for the complete disintegration of 0·7 gm. yeast; here 2 gm. yeast was completely disintegrated in 4·5 minutes. The new shaker is cheap to build, easy to maintain and repair. Disintegrations are rapid and the percentage disintegration moderately reproducible: Foulkes (1951) was unable "to standardize fully the extent of cell disintegration" (p. 379).

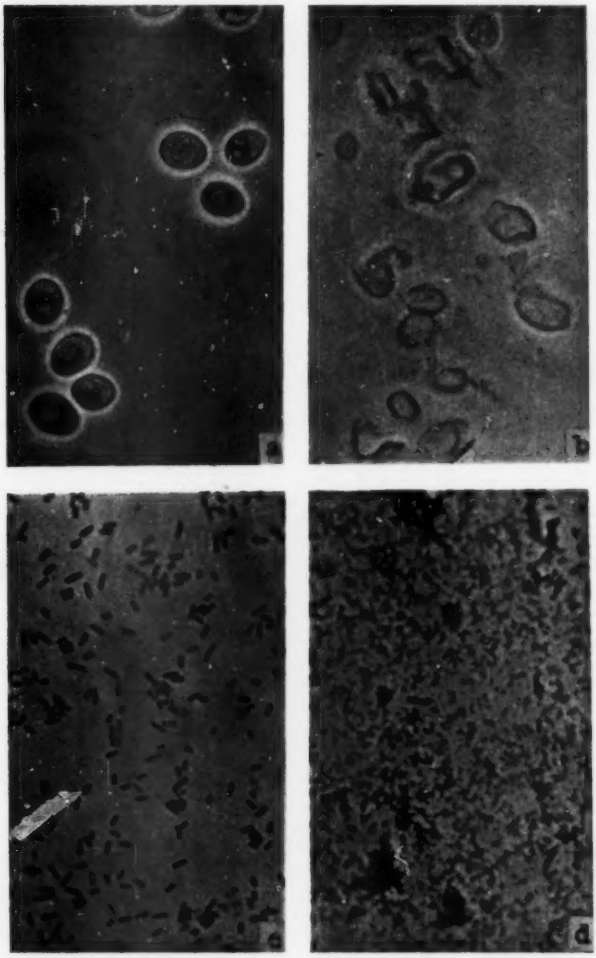

Fig. 2. Photomicrographs of cells before and after disintegration. Zeiss phase contrast; oil immersion (900 × magnification); photographed with a Leica camera on Ilford FP 3 and enlarged two-fold on to Kodak F 2 Bromide.

(a) Wet preparation of fresh *Saccharomyces cerevisiae.*

(b) The same after disintegration.

(c) Dry preparation of *Proteus vulgaris,* stained with crystal violet.

(d) The same after disintegration.

TABLE 2.

Comparison of three dehydrogenase activities in frozen and disintegrated yeast cells.

Substrate	$Q^*_{M.B.} \times 100$	
	Lynen and Neciullah (10)	Present work
Blank	<35	<40
Succinate	110	1,000
Blank	80	<40
Malate	140	1,400
Blank	100	<40
Citrate	250	830

* = μl. methylene blue decolourized/mg. dry weight/hour, calculated from the published data, on the assumption that the wet weight/dry weight ratio of Lynen and Neciullah's yeast was also *ca.* 3/1.

Cell-free yeast extracts prepared with the new shaker show higher activities for certain enzymes than have ever been reported in intact or treated yeast cells. The highest recorded $Q_{fumarase}$ value (Krebs *et al.*, 1952) was 75 for frozen yeast cells; the activities obtained here were 10–20 times greater. The three dehydrogenase activities studied in liquid N_2-frozen cells (Lynen and Neciullah, 1939) were much lower than in extracts (Table 2). Probably even frozen cells are only partially permeable to the ions of dicarboxylic acids (Nossal, 1951c). Permeability barriers may account for the low activities (Krebs, 1949; Krebs *et al.*, 1952) or negative results (Kleinzeller, 1941) reported for Krebs cycle enzymes, which occur in yeast cells in much higher concentrations than previously suspected. The work with cell-free extracts is being continued.

SUMMARY.

A mechanical shaker is described, which performs 5,600 r.p.m. with an amplitude of 5/8″.

2 gm. *Saccharomyces cerevisiae* are totally disintegrated by 4·5 minutes shaking with Ballotini glass beads, and 0·5 gm. *Proteus vulgaris* or *Lactobacillus arabinosus* in *ca.* 2 minutes.

Shaking conditions may be carefully controlled and the percentage disintegration for a given shaking period is fairly reproducible.

Q values are given for the fumarase, aspartase, pyruvic and acetolactic decarboxylase activities of various extracts. Seven yeast dehydrogenases are studied and their activities in the extracts shown to be much higher than those observed in treated yeast cells.

Acknowledgments. The author is indebted to Mr. V. Ingerson for constructing the shaker, to Professor H. H. Davis for mechanically improving the design, to Mr. G. Gormley for drawing Fig. 1, to Messrs. P. A. Trudinger and A. L. Hunt for the data on *Proteus* and *Lactobacillus* extracts and to Drs. W. R. Adey and D. Packer for the use of photomicrographic equipment.

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SALMONELLA BACTERIOPHAGES

II. BACTERIOPHAGES OF *S. WAYCROSS*

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Atkinson *et al.* (1952) reported the occurrence of two lysogenic strains of *S. waycross* among 31 tested. The phages from both strains appeared similar and their general characteristics are given in the present paper.

METHODS.

The phages were isolated by cross-plating with strain Currie and subsequently purified by picking single plaques from successive platings on strain Currie. Heat resistance tests and serological tests were performed as described by Atkinson and Geytenbeek (1953) for *S. adelaide* phages except that patch tests instead of plaque counts were used to test the treated phage preparations. Resistant strains were obtained by growing strain Currie in broth overnight with the phage, plating out the resultant growth and testing separate colonies for susceptibility to lysis by phage and for lysogenicity. To detect lysogenicity the test strain was cross-plated with strain Currie, the spot of mixed growth was extracted with a little broth which was centrifuged and then patch tested on strain Currie and the test strain.

RESULTS.

Isolation and range of action of the phages. Phages were isolated from the two lysogenic strains Cronin and S 215 and labelled W Cr and W 215 respectively. The plaques of each phage were similar but rather heterogeneous in size and extent of secondary growth. However, these variations appeared in single plaque preparations from plaques of various sizes and degrees of secondary growth and seemed to represent various stages in development of the plaques. When tested on our 31 strains of *S. waycross* (described by Atkinson *et al.*, 1950), phages W Cr and W 215 lysed all strains except Cronin and S 215 from which they were derived. The 29 strains which were lysed all came from a single outbreak of gastro-enteritis and were subsequently called *S. waycross* Atherton group (see Table 2). Both phages were also tested on a number of Salmonellas representing various O groups and on other members of the *Enterobacteriaceae*. The results are given in Table 1.

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TABLE 1.

Reactions of S. waycross *phages with other members of the* Enterobacteriaceae.

Organism tested	No. of strains	Phage used in test	
		W Cr	W 215
S. paratyphi A	1	—	—
S. typhi-murium	2	—	—
S. derby	3	—	—
S. muenchen	2	—	—
S. bovis-morbificans	5	—	—
S. bloqdam	2	—	—
S. paratyphi B	2	—	—
S. adelaide	1	—	—
S. enteritidis	2	—	—
S. anatum	2	—	—
S. worthington	2	—	—
S. aberdeen	1	—	—
S. onderstepoort	1	—	—
S. carrau	1	—	—
S. waycross (strain Currie)	1	+	+
Arizona NJ4	1	—	—
E. coli O 111	1	—	—
E. coli O 55	1	—	—
Ballerup	1	—	—
Bethesda	2	—	—
Sh. flexneri	1	—	—
Sh. boydii	1	—	—
Sh. dispar	1	—	—
Sh. sonnei	1	—	—
Proteus	2	—	—

Phage preparations used undiluted. + = confluent lysis. − = no lysis.

The organisms tested here were identical with those used in similar tests with *S. adelaide* phages (Atkinson and Geytenbeek, 1953). The *S. waycross* phages failed to attack any of the *Enterobacteriaceae* tested except *S. waycross* Atherton group represented by Currie, the propagating strain. The Arizona paracolon NJ4, with O antigenic formula similar to *S. waycross,* was not lysed by either phage.

Plaque and patch characters. The plaques and patches of W Cr and W 215 were similar. The plaques were small (average diameter 1 mm.) with irregular edge and large central spot of secondary growth. The patches of confluent lysis resembled the plaques and showed a large central disc of secondary growth surrounded by a small moderately clear ring with irregular edge.

Heat resistance. At a dilution producing semi-confluent lysis, the activity of phages W Cr and W 215 was slightly reduced after 30 minutes at 65° C. and no lysis or a few plaques appeared after 30 minutes at 70° C.

Serological tests. Antiserum was prepared against phage W Cr and at 1/100 it completely neutralized dilutions of phage W Cr producing confluent lysis in

the control. Similar preparations of phage W 215 were also neutralized by 1/100 W Cr antiserum. The phages were thus serologically identical.

Production of resistant strains and lysogenicity. Strain Currie was treated with either phage W Cr or phage W 215 in broth. Numerous resistant colonies were obtained with both phages. All resistant strains tested were lysogenic.

Brief summarized description of S. wayeross *phage.* The two phages W Cr and W 215, so far found in lysogenic strains of *S. waycross,* appeared to be identical. We suggest that they be designated *S. waycross* phage type 1, and the type strain be phage W Cr. A brief description follows.

S. waycross phage type 1. Plaques with irregular edge and large central spot of secondary growth, moderately heat resistant (to 65° C. for 30 minutes), inactive on numerous other Salmonella types as well as other members of the *Enterobacteriaceae,* readily produced resistant, lysogenic strains from susceptible strains of *S. waycross,* found in naturally occurring lysogenic strains of *S. waycross* group 1 (see Table 2), lysed strains of *S. waycross* Atherton group.

Phage grouping of strains of S. wayeross. The 31 strains of *S. waycross* fell into two groups according to lysis or lysogenicity. The grouping set out in Table 2 is therefore suggested.

TABLE 2.

Phage grouping of strains of S. wayeross.

S. waycross grouping	Reaction with phage type 1	Phage type carried
Group 1 (strain Cronin)	—	type 1
Atherton group (strain Currie)	+	none

S. waycross group 1 carried phage type 1 and was resistant to lysis by this phage. Two strains, Cronin (from South Australia) and S 215 (type strain from U.S.A.) belonged to group 1. *S. waycross* Atherton group was lysed by phage type 1 and was not lysogenic. Twenty-nine strains from a single outbreak of gastro-enteritis on the Atherton Tableland, Queensland (described by Ferris and Hertzberg, 1945), belonged to this group.

DISCUSSION.

Both the lysogenic strains of *S. waycross* carried the same phage which has been designated type 1 and is the only *S. waycross* phage so far found. It lysed

all strains of the Atherton group of *S. waycross*. Its range of action was most restricted, however, as it failed to lyse any other member of the *Enterobacterioceae* tested. These tests covered an extensive range of Salmonella types as well as Shigellas and members of Ballerup, Bethesda, Arizona, Proteus and Escherichia groups. Thus the only strains of *Enterobacteriaceae* lysed by the type 1 *S. waycross* phage were those of the Atherton group of *S. waycross*. Without strains of this group, the phage could not have been detected. Had we possessed only the type strain (S 215) and our South Australian strain, lysogenicity would not have been detected by either of these strains or by any other Salmonella so far tested. This is a striking and simple example of the difficulties which may confront those who search for the phage of lysogenic strains.

The grouping of the 31 *S. waycross* strains on phage carriage and phage lysis correlated well with the origin of the strains. The Atherton strains, which were all isolated from cases occurring in a single, short epidemic, were similarly susceptible to the type 1 phages and failed to reveal any phage themselves. They thus formed, as might be expected, a homogeneous group of epidemiological significance, the Atherton group. The other two strains were similar but unrelated epidemiologically.

SUMMARY.

From two lysogenic strains of *S. waycross* identical bacteriophages have been isolated and designated *S. waycross* phage type 1. The characteristics of the phage are given and on the basis of phage carriage and phage lysis 31 strains of *S. waycross* have been divided into two groups, group 1 carrying phage type 1 and resistant, and the Atherton group not carrying phage type 1 and sensitive to this phage.

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THE AMINO-ACID COMPOSITION OF CERTAIN SEED PROTEINS

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A survey of the literature based on the accumulated data of Block and Bolling (1951) indicates that knowledge of the amino-acid composition of the proteins of legume seeds is limited. There is an almost complete lack of comprehensive data for the seeds of legumes of particular importance to Australian agriculture, such as lupins, vetches, peas and subterranean clover. Investigations of the digestibility and biological value of the protein of some of these seeds, using nitrogen balance methods, have been reported from this laboratory for the growth of sheep (Williams and Moir, 1951) and for the growth of the rat (Smythe, 1950) but they were not supported by any extensive data on their amino-acid composition. The arginine content of these seed proteins was, however, reported by the author (Holmes, 1951) and the cyst(e)ine and methionine contents of some of them by Johanson (1948).

In recent years considerable attention has been devoted to the sulphur-containing amino-acids in seed proteins (Lugg, 1945 and 1946; Johanson and Lugg, 1946). As a result it has been established that legume seeds and their bulk proteins are of very low or low methionine content and their cyst(e)ine values range from very low to moderately high. Numerous feeding trials indicate that the methionine contents of legume seeds may constitute a first limiting factor in nutrition, at least in the rat and the chick. On the other hand, in spite of the not unexpected emphasis placed on the sulphur-containing amino-acids in wool growth (Marston, 1935) no correlation could be found by Johanson *(loc. cit.)* between cyst(e)ine + methionine fed and wool growth in the series of feeding trials carried out by Stewart and Moir (1947). In these trials marked differences were found between different protein sources, including several legume seeds, in promoting wool growth in Merino sheep when these proteins were fed at the same overall level of nitrogen intake and constituted almost the whole of the nitrogen of otherwise similar rations. The possibility of other essential amino-acids limiting the wool-growing capacity of these proteins, particularly those occurring in wool keratin in high proportions, namely arginine, leucine and threonine, could not be excluded. The present study of the amino-acid composition of certain legume seed proteins and of linseed protein,

meatmeal protein and the proteins of oaten chaff was therefore undertaken, not only to obtain information about these materials of general nutritional importance but also with the hope that they might illuminate the feeding trials on wool growth.

Materials and Methods.

(a) *Materials.* Seed materials selected for analysis were those of Lupin (*Lupinus pilosus.* W.A. blue), Pea (*Pisum sativum.* var. White Brunswick), Subterranean Clover (*Trifolium subterraneum.* var. Dwalganup), Linseed (*Linum usitatissimum*) and Wheat (*Triticum vulgare*). Samples of meatmeal and oaten chaff were also examined.

(b) *"Whole" protein preparations.* Whole protein preparations were made from the ground material by the method of Lugg (1939), with Lugg and Weller's (1944) modification. This process involved denaturation and precipitation of the proteins present and removal of non-protein nitrogenous compounds and some other substance, whilst retaining a certain amount of carbohydrate material. Nitrogen determinations were made by the micro-Kjeldahl method of Willets *et al.* (1949) using mercuric oxide as a catalyst.

(c) *Protein hydrolysis.* For the estimation of tryptophane, alkaline hydrolysis of the protein preparations was carried out with 5 N NaOH in sealed test tubes at 100° C. for 24 hours. Following filtration the solution was neutralized with H_2SO_4 and made up to a volume of 20 ml. This neutralized hydrolysate was desalted prior to the chromatographic separation of the amino-acids by the use of the electrolytic desalting apparatus designed by Consden, Gordon and Martin (1947), except that the carbon anode was replaced with platinum (de Verdier and Agren, 1948). Tests with standard tryptophane salt solutions gave a 91 p.c. recovery of tryptophane by this method. The final dilute desalted solutions were then concentrated in a vacuum desiccator.

Acid hydrolysis was used for the rest of the amino-acids to be determined. The protein preparations were hydrolysed with freshly distilled constant boiling HCl (5·7 N) in sealed test tubes at 105° C. for 24 hours. The quantity of protein preparation taken (200-500 mg.) was such as to give a final concentration of approximately 5 mg. N/ml. with an acid to protein ratio of 10 ml./gm. After filtering off the humin formed, the excess acid was removed under reduced pressure. The resulting amino-acid deposit was taken up in 5 ml. of distilled water, slightly acidified to retain the less soluble amino-acids in solution.

(d) *Chromatographic technique.* The general methods adopted for the paper chromatographic separation of the amino-acid constituents were based on the report of Consden, Gordon and Martin (1944). For descending solvent movement air-tight galvanized iron tanks were used, the filter paper being suspended from stainless steel troughs. For ascending solvent migration cylindrical glass museum jars were used in the manner of Williams and Kirby (1948). Whatman No. 4 filter paper was used throughout. Phenol was purified by the method of Williams and Kirby (1948), collidine by the method of Partridge (1948) and alcohols by fractional distillation. Water saturated solvents were allowed to come to equilibrium until clear solutions were obtained at the chromatographic temperature. To avoid losses of amino-acids associated with excessive heating in the presence of solvents (Fowden and Penny, 1950) phenol was removed from the chromatogram by ether washing, collidine by drying in a current of air at 50° C. and alcohols by air drying at room temperature. The dried papers were heated for a further 15 minutes at 100° C. and the positions of the amino-acids marked out under ultra-violet light (Phillips, 1948). Methionine, leucine, *iso*-leucine and phenylalanine were satisfactorily separated by the method of Work (1949) on a uni-dimensional scale in the descending manner with water-saturated amyl alcohol as the solvent. The remaining amino-acids were separated on a two-dimensional scale using two solvent systems, namely, the descending method of Consden *et al.* (1944) with water saturated phenol (1 p.c. NH_3) and

collidine and the ascending method of Boissonnas (1950) with phenol-water (7: 3) and propanol-water (7: 3).

(e) *Amino-acid estimations.* Quantitative estimations of the amino-acids were based on the reports by Fowden (1951) and Boissonnas (1950) who successfully adopted the ninhydrin reagent of Moore and Stein (1948) to a spectrophotometric estimation from papergrams. Standard reference graphs, relating amino-acid nitrogen to optical density, were drawn up for each amino-acid by the use of standard amino-acid solutions and the linear plot obtained was shown to be reproducible with an accuracy of ±2 p.c. By reference to this standard curve quantitative estimations were obtained from the chromatograms with an accuracy assessed at ±5 p.c.

RESULTS.

(a) *"Whole" protein preparations.* Data concerning the fresh materials and the protein preparations made from them are given in Table 1.

TABLE 1.

Details of "whole" protein preparations.

Seed material	Fresh wt. (gm.)	P.c. moisture	Dry wt. (gm.)	P.c. N.	Total N (mg.)	Protein N (mg.)	Dry wt. protein prep. (gm.)	P.c. N. protein prep.
*Sub-clover	29·91	5·48	28·36	5·64	1,599	1,224	17·21	7·11
*Linseed	30·33	6·54	28·35	6·30	1,786	1,477	22·61	6·53
*Vetch	32·97	9·52	29·83	4·52	1,348	1,147	26·25	4·37
*Pea	31·93	11·1	28·38	4·55	1,291	1,164	23·96	4·44
*Lupin	30·73	10·2	27·60	5·47	1,510	1,371	23·96	5·72
Meatmeal	10·62	8·28	9·85	8·90	876·6	732·2	7·25	10·1
Oaten chaff	10·00	10·9	8·91	0·91	81·1	73·1	6·90	1·06

* Data previously published (Holmes, 1951).

Consideration of the data of Table 1 provides an interesting comparison of the relative amounts of non-protein nitrogen (N.P.N.) and carbohydrates and other substances removed during the extraction. The subterranean clover seed sample contained a large amount of extractable non-protein material compared with the other seed samples. This is in conformity with the values reported by Lugg and Clowes (1945), who found the total N percentage to be 5·80 and the percentage of N in the protein preparation to be 7·69 for the sample of seed examined. In addition, it can be seen that the proportion of non-protein nitrogenous substances to the other extractable substances with the vetch and pea preparations is large compared with some other seed preparations (see also Lugg and Weller, 1944, and Lugg and Johanson, 1946). The per cent. N in the "whole" protein preparations in these cases is lower than in the unextracted seeds.

(b) *Hydrolytic destruction of amino-acids.* A variable destruction of amino-acids during acid hydrolysis of protein materials, particularly where these materials contain carbohydrate, has been reported by a number of workers. Experience in this laboratory suggests that the extent of this destruction has

generally been underestimated, as the following experiment shows. A mixture of pure amino-acids was heated with 5·7 N HCl and a quantity of arabinose in a sealed tube at 105° C. for 24 hours and the recovery of each acid determined chromatographically, as described earlier. The amino-acid/arabinose ratio was based on a protein preparation of 5 p.c. N, so that assuming a value of 16·5 p.c. N for a hypothetically pure protein preparation, the impurity in 500 mg. of protein preparation was represented by 350 mg. of arabinose. To this quantity of arabinose the amounts of amino-acids present in 500 mg. of a typical seed protein preparation were added. The resulting amino-acid destructions, including that for tryptophane during alkaline hydrolysis, are given in Table 2.

TABLE 2.

Hydrolytic destruction of amino-acids.

	Percentage destruction	Correction factor
Arginine	37·6	1·60
Histidine	29·0	1·41
Lysine	31·0	1·45
Threonine	12·6	1·14
Valine	22·0	1·27
Methionine	27·8	1·38
Phenylalanine	5·9	1·06
Leucine	13·4	1·15
Iso-leucine	15·7	1·18
Tyrosine	25·0	1·33
Tryptophane	7·4	1·08
Alanine	11·8	1·13
Aspartic acid	4·6	1·05
Glutamic acid	6·8	1·07
Glycine	11·9	1·13

The hydrolytic losses presented in Table 2 are in relative conformity with those obtained for many different amino-acids by other workers (Tristram, 1939 and 1940; Borchers, Totter and Berg, 1942; Lyman *et al.*, 1943; Block and Bolling, 1951) but the actual values are higher, particularly with arginine, histidine, lysine, methionine and tyrosine.

How far these results are directly applicable to the protein preparations being investigated is, of course, not known, especially as the method of assessment used involves the assumption that free amino-acids react in the same way during treatment as their analogues in peptide linkage. But the procedure adopted is the best approximation that can be made at the present time and the conditions of hydrolysis actually used were chosen to conform, as far as possible, to those believed to occur during hydrolysis of the protein preparations under study.

(c) *Amino-acid composition of protein preparations.* The results of the amino-acid analyses, expressed as a percentage amino-acid nitrogen of protein nitrogen and corrected for hydrolytic losses, are recorded in Table 3. The losses

involved in hydrolysis of the protein material were compensated for by multiplying the actual analytical figures by the appropriate "correction factor" for each amino-acid, taken from Table 2.

TABLE 3.

Amino-acid composition of "whole" protein preparations.
Per cent. amino-acid N of protein N.

Amino-acid	Vetch seed	Lupin seed	Pea seed	Sub. clover seed	Linseed	Meat-meal	Oaten chaff	Whole egg protein*
Arginine	24·8	23·5	21·6	23·5	24·3	24·0	21·4	12·9
Histidine	8·9	10·0	7·8	5·3	7·1	6·4	5·2	3·6
Lysine	6·8	7·6	9·7	11·0	11·9	8·5	8·4	8·6
Threonine	3·8	3·3	3·0	1·3	3·9	2·3	2·7	3·6
Valine	5·9	5·5	3·1	3·0	4·9	3·9	4·9	5·5
Leucine	4·8	7·4	5·3	3·1	5·0	6·5	5·5	6·1
Iso-leucine	3·8	4·9	3·2	1·8	2·6	2·8	4·0	5·3
Phenylalanine	4·1	4·0	3·0	1·9	2·8	2·7	3·3	3·3
Tryptophane	0·9	1·9	1·7	1·6	2·1	—	—	1·3
Methionine	1·5	1·1	1·0	0·9	1·4	1·7	1·2	2·4
Tyrosine	3·4	3·5	2·8	1·1	2·1	2·5	1·5	2·2
Alanine	3·5	2·7	3·7	4·0	6·3	5·6	4·7	—
Aspartic acid	8·8	6·7	5·7	5·6	6·3	5·8	6·6	—
Glutamic acid	10·4	14·8	18·9	13·8	15·6	17·9	12·2	—
Glycine	4·5	4·6	7·1	4·0	4·5	6·2	8·1	2·6

* Block and Bolling (1951).

It is apparent from the data of Table 3 that the greatest variation between the materials studied occurs in the contents of the non-essential amino-acids, alanine, tyrosine, glycine and glutamic acid. The basic amino-acid contents of all samples are high and of the same order and the arginine contents are in reasonable agreement with those previously reported by the author (Holmes, 1951) using an entirely different method of analysis.

The methionine values are, however, very much higher than those previously reported for comparable materials by Johanson (1948), who used the fairly rigorously tested differential-oxidation method of Lugg (1938). To some extent this difference can be accounted for by the large "correction" factor used (1·38) to compensate for the estimated hydrolytic loss. But even when the methionine values are presented with no correction for hydrolytic loss, several of them are still appreciably higher than those of Johanson, Lugg and others, as the following figures show:

	Vetch	Pea	Lupin	Sub. clover	Meatmeal
Holmes ("corrected")	1·5	1·0	1·1	0·9	1·7
Holmes ("uncorrected")	1·1	0·8	0·8	0·7	1·3
Johanson (1948)	0·7	0·7	0·5*	0·3	1·2†

* Embryo only. † Block and Bolling.

In the present study the methionine estimations were near the lower limit of the spectrophotometer, which did not apply to the rest of the amino-acids other than tryptophane. This may provide some explanation of the high methionine values. On the other hand, the total recovery of the N for the 15 amino-acids studied, using the "corrected" values, is very close to 100 p.c. for several of the materials. In such materials serine, cyst(e)ine and amide should represent an appreciable fraction of the total N, possibly of the order of 10–15 p.c. As it is unlikely that the values recorded are uniformly high by this proportion it is probable that one or more of them, including methionine, are definitely too high, principally because of over-compensation for hydrolytic loss. This point will be taken into consideration in the ensuing discussion on the nutritive value of the feeds studied.

DISCUSSION.

Mitchell and Block (1946) have proposed a method of assessing the nutritive value of various protein sources by comparing their essential amino-acid contents with those of whole egg protein, which is almost perfectly utilized by the growing rat as a source of N and is therefore assumed to have a practically perfect assemblage of these amino-acids. The essential amino-acid which is present in least proportion compared with the amount in whole egg protein is designated as the "limiting" amino-acid and the "percentage deficit" of individual amino-acids can be calculated. The sum of the individual percentage deficits is called the "total percentage deficit" of the protein.

Applying this method to the protein materials under study and using the presumably high methionine values recorded in Table 3 it can be shown that the limiting amino-acid with linseed and meatmeal is, in each case, *iso*-leucine ("percentage deficit" —52 and —49 respectively) followed by methionine (—40) with the linseed and threonine (—36) with the meatmeal. In the leguminous seeds methionine is the limiting amino-acid with lupin (—55), vetch (—38) and pea (—57), whilst the subterranean clover seed is severely and almost equally deficient in methionine (—62), *iso*-leucine (—66) and threonine (—64). *Iso*-leucine, it should be pointed out, is also deficient in the vetch and pea seed proteins (percentage deficit —30 and —40 respectively). Pea seed protein is also very deficient in valine (—43) compared with whole egg protein. The oaten chaff protein is, with the lupin, the most complete of those examined, being only deficient in methionine (—50) and slightly deficient in *iso*-leucine (—26) and threonine (—25).

These results confirm the comparative methionine deficiency of the legume seeds, which has been established by a number of workers, a point of considerable interest in the light of discussion in the preceding section emphasizing that the levels given in Table 3 for this amino-acid are almost certainly too high. If the levels of methionine found in similar materials by the use of other methods had been adopted the limiting nature of this amino-acid would have been still more apparent.

The results reveal the further important fact, which so far as is known has not previously been observed, that *iso*-leucine is also generally deficient in leguminous seeds and ranks next to methionine in significance. This remark applies also to linseed and meatmeal. Finally, the extremely poor supply of essential amino-acids in subterranean clover seed protein, compared not merely with whole egg protein but also with all the other protein sources included in this study, deserves some mention. The "total percentage deficit" in subterranean clover seed is no less than —331, compared with —178 for pea seed, which is the poorest of the rest, and with —86 for lupin seed the best of the group.

Mitchell and Block *(loc. cit.)* were able to demonstrate a distinct correlation between the biological value of many food proteins and their per cent. deficits in the limiting amino-acids; that is, by listing the protein materials in increasing deficiency of the limiting amino-acids it was found that a general decrease in biological value was recorded. For several of the protein sources in the present study no biological values obtained with rats have been recorded, nor have studies been made of the supplementary value of particular amino-acids. Such investigations are necessary before a proper assessment of the results presented in this paper can be made in nutritional terms. The biological values of linseed and subterranean clover seed proteins determined in growing lambs by Williams and Moir *(loc. cit.)* do not support the suggestion of a marked inferiority for subterranean clover seed protein but, as Holmes, Moir and Underwood (1953) have recently pointed out, biological value in ruminants is not merely a reflection of the contents of essential amino-acids in the protein, it is influenced also by the extent of conversion to rumen bacterial protein and of rate of deamination by the rumen micro-organisms with possible loss of ammonia to the host animal.

SUMMARY.

Fifteen amino-acids were determined, by paper chromatography, on the "whole" protein preparations of the seeds of the lupin *(Lupinus pilosus)*, pea *(Pisum sativum)*, vetch *(Vicia sativum)*, subterranean clover *(Trifolium subterraneum)*, linseed *(Linum usitatissimum)* and wheat *(Triticum vulgare)*, and of commercial samples of meatmeal and oaten chaff.

Variation in the proportion of non-protein nitrogen and other extractable substances was found with the seeds. The non-protein nitrogen fraction ranged from one-tenth to one-fifth of the total nitrogen present.

Evidence was presented that considerable destruction of the amino-acids arginine, histidine, lysine, methionine and tyrosine occurs during acid hydrolysis and "correction factors" have been used in an attempt to compensate for such losses.

Compared with whole egg protein as a standard the amino-acids limiting the nutritive value of the seed proteins were found to be as follows: lupin—methionine; vetch—methionine and *iso*-leucine; pea—methionine, *iso*-leucine and

valine; subterranean clover seed—*iso*-leucine, threonine and methionine; linseed—*iso*-leucine and methionine.

The comparative methionine deficiency of legume seeds, shown by earlier workers, was therefore confirmed and a general *iso*-leucine deficiency in these materials also established.

Based on the degree to which the essential amino-acid contents differed from those of egg protein, the nutritive value of the protein sources examined was found to decrease in the following order: lupin, vetch, linseed, meatmeal, pea and subterranean clover. The subterranean clover seed protein was found to be severely and almost equally deficient in *iso*-leucine, threonine and methionine.

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ACCURACY OF BLOOD CELL COUNTING

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It is commonly believed that in a series of blood counts, performed with adequate technique, the accuracy of counting can be improved by rejecting counts in which the distribution of cells appears unduly irregular. Since this belief does not appear ever to have been justified by a critical experiment nor does it rest on any theoretical basis, it has been tested here by an experiment.

The whole of the ruled area of the haemocytometer chamber has been counted. The counts on blocks of 1/25 square millimetres have been recorded and gathered into sets of five. Measures of the dispersion in the sets and of the accuracy of the set totals have been defined. The dispersion and accuracy are found not to be correlated. It is assumed, therefore, that much of the advice given to technicians on the counting of blood cells is justifiable neither by theory nor by experiment and that the deliberate selection of squares does not improve the accuracy of the counts. The practice is highly undesirable on other grounds and should be abandoned.

The Measures of Dispersion and Accuracy.

The measure of dispersion within a set has been taken to be

$$\chi^2 = \Sigma\,(x-\bar{x})^2/\bar{x} \qquad (1)$$

where $\bar{x}$ is the mean of the counts, x, in a set. The quantity on the right of (1) might still be used as a measure of dispersion even if the mean count per small haemocytometer square were high and its distribution no longer that of χ^2. However, at the level of the mean counts, used in this paper, the χ^2 distribution may be considered appropriate (Lancaster, 1950). The sets were ranked in order of accuracy by taking the absolute difference of each mean from the grand mean of the experiment. Spearman's coefficient of rank correlation, ρ, was used as the appropriate test of the independence of the rankings and the significance was tested by writing

$$t = (n-2)^{\frac{1}{2}}\rho/(1-\rho^2)^{\frac{1}{2}} \qquad (2)$$

and testing t as Student's t with $(n-2)$ degrees of freedom.

Method.

A Neubauer haemocytometer of the older design was used in the author's counts, so that the whole of the two square millimetres could be counted. A dilution of blood was chosen so that the mean was approximately 1·5 cells per small haemocytometer square. Three complete counts of both haemocytometer chambers formed a single experiment. Three such experiments were carried out by the author, one by Dr. Walsh and one by Dr. Mattocks.

RESULTS.

The results of the author's first experiment are set out in Table 1.

TABLE 1.

Experiment 1. The rankings of a set of counts by accuracy and by dispersion.

Counts on blocks					Set total	Ranking by distance from mean	Difference of (7)—(9)	Ranking by χ^2	Value of χ^2 for set
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
24	14	31	25	32	126	12	—16	28	8·21
17	26	34	19	29	125	14	—13	27	7·92
26	18	28	32	31	135	13	—9	22	4·59
26	32	21	23	32	134	11	—7	18	3·84
22	26	28	27	33	136	15	6	9	2·31
37	41	21	25	20	144	26	—4	30	12·81
24	27	25	20	32	128	6	—5	11	3·02
39	30	32	29	24	154	30	11	19	3·86
24	31	19	25	18	117	24	1	23	4·67
29	26	24	32	26	137	16	12	4	1·43
21	28	24	27	30	130	1	—6	7	1·92
33	18	23	21	23	118	23	—1	24	5·39
28	19	32	29	24	132	5	—12	17	3·83
26	32	27	31	21	137	17	7	10	2·82
31	29	21	32	24	137	19	5	14	3·26
31	23	31	22	34	141	22	1	21	4·07
20	23	26	27	27	123	20	15	5	1·51
19	21	24	24	19	107	29	26	3	1·18
33	31	27	32	24	147	27	19	8	1·95
28	17	31	21	31	128	8	—18	26	6·22
26	20	23	22	21	112	28	26	2	0·95
23	28	24	18	29	122	21	8	13	3·16
28	27	32	19	26	132	4	—12	16	3·38
21	31	25	21	29	127	10	—5	15	3·28
33	22	25	35	22	137	18	—7	25	5·59
38	23	17	21	29	128	7	—22	29	10·44
24	33	24	31	21	133	9	—11	20	3·95
25	22	31	31	22	131	3	—9	12	3·16
26	24	21	25	21	117	25	24	1	0·91
27	21	30	25	28	131	2	—4	6	1·79

The manner of obtaining the rankings is evident from the Table. χ^2 was computed to four decimal places and there were no ties. Where ties in accuracy occurred, the ranks are given in the Table after a random sampling auxiliary experiment. In Table 2 are summarized the results of the three counters. The coefficient, ρ, is usually negative and once significantly so. The results are not in favour of the common assumption that the accuracy and the dispersion are positively associated in any group of counts carried out with an adequate technique.

TABLE 2.

Identification of experiment	Number of sets of five counts	Mean number of cells per set	Coefficient of rank correlation	Value of t	Probability of t using a "one-way" test
1, (L)	30	130·2	—0·107	—0·57	0·72
2, (L)	30	127·6	—0·383	—2·13	0·98
3, (L)	30	84·9	0·240	1·29	0·10
4, (W)	30	167·4	—0·162	—0·87	0·81
5, (M)	30	216·8	—0·044	—0·23	0·59

DISCUSSION.

The foregoing methods have been applied to other reported series of counts such as those of Lancaster (1950), Plum (1936), Bürker (1905 and 1911) and Bryan and Garrey (1934) but no consistent positive association between the accuracy and the dispersion has been demonstrated. A full analysis has been reported in a thesis (Lancaster, 1953). Some authors, such as Bryan and Garrey (1934), have laid great stress on the necessity for a long shaking, although Magath, Berkson and Hurn (1936) were unable to confirm their results. The author's counts of this set of experiments have been analysed with a view to detecting changes in dispersion, but no evidence of heterogeneity in the dispersion has been found; so that the dispersion has been presumed to be optimal.

SUMMARY.

Measures of the dispersion and of the accuracy of a set of counts have been defined. In experiments carried out with satisfactory technique, there is no correlation between the two measures. Further evidence on this point has been obtained by the analysis of published results.

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THE EFFECT OF ADRENAL ABLATION ON THE MUSCLE ACTION POTENTIAL OF RATS[1]

by G. W. MILTON

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(Accepted for publication 8th July, 1953.)

The endocrine influence on muscle function has not been extensively investigated with the electromyograph and the present study was designed to test the influence of adrenal ablation on the duration of muscle action potentials in the rat.

METHODS.

Adult male albino rats weighing between 150 and 200 gm. were used throughout this investigation. The animals were anaesthetized by open ether. Two stimulating electrodes were implanted, one on either side of the vertebral column in the lower thoracic region. The electrodes must be deeply placed to ensure that a current passed across them will stimulate the spinal cord. The stimulator gives a short impulse (0·1 millisecond) which is synchronized to the sweep of a cathode ray oscilloscope. The stimulus used was the minimal strength required to produce a tetanic twitch of the muscles caudal to the stimulating electrodes, usually less than ten volts.

A concentric needle electrode similar to that first described by Adrian and Bronk (1929) was used for recording throughout these experiments. It consisted of a size 21 hypodermic needle down the centre of which was passed a fine insulated silver wire (36 S.W.G.). The bevel was 0·6 mm. diameter and the central wire protruded 0·05 mm. beyond the end of the bevel. The same recording electrode was used in all experiments, because Petersen and Kugelberg (1949) have shown that the duration of the recorded action potential is influenced by the size of the recording electrode.

Through a short incision on the anterolateral aspect of the thigh the fascia covering the quadriceps femoris muscle was cleared and the recording electrode was inserted into the muscle belly. After each action potential had been photographed the recording electrode was removed and reinserted in a different position. In this way, ten action potentials of different wave forms were recorded at each session. To avoid excessive trauma to these small muscles, each was used on only two occasions; that is, not more than twenty recordings were taken in all from any one muscle. At alternate sessions the opposite leg was used and in this way each animal could be tested a maximum of four times.

To check the validity of the method a series of intact and conscious animals was investigated. The rat was anaesthetized and the recording electrode inserted into the quadriceps femoris. The animal was then allowed to regain consciousness. Left alone the rat would lie quietly, not giving any evidence of pain, but if its feet, tail or nose were touched it would struggle to be free. The duration of the action potentials obtained from this voluntary movement were compared with those obtained from anaesthetized and stimulated animals.

Bilateral adrenalectomy was performed through a transverse ventral abdominal incision, both glands being removed at a single operation. The ventral approach was preferred to

[1] The expenses of this research were borne by a grant from the Medical Research Committee, University of Adelaide.

Grollman's posterior method (Grollman, 1936a) because it facilitates excision of the adrenal gland and perirenal tissue in one block, thereby permitting the removal of any accessory adrenal tissue and decreasing the chance of seeding cortical cells, which could regenerate, in the wound. After adrenalectomy the rats were weighed daily and any animal that did not lose weight or die, or which at autopsy showed any tissue proven histologically to be adrenal substance, was discarded from the series. Animals kept alive by adrenocortical extracts or saline drinking water were tested, after the withdrawal of these substances, by the same criterion to show that total adrenalectomy had been performed.

One group of five normal rats was given potassium chloride injections intraperitoneally, 8 ml. of a 1 p.c. solution given 120, 60 and 30 minutes before the E.M.G. A similar group was also given the same dose of sodium chloride (1 p.c.) and another group calcium chloride (2 p.c.) by the same route.

Bilateral nephrectomy was performed on four rats through the lumbar incision described by Grollman (1936a) for adrenalectomy. The kidney was carefully shelled out of the perinephric fat to avoid damage to the adrenal glands. The specimen was examined to show that no adrenal tissue had been removed and at autopsy the adrenals were seen to be intact. The electromyograph of these animals was investigated 48 hours after nephrectomy.

In the early stages of this work a sensitive mercury thermometer was strapped along the rat's belly with the bulb lying between the hind legs. But it was soon apparent that the small variations in body temperature that occurred did not affect the duration of the action potential, and so this precaution was abandoned.

To estimate the effect of acute anoxia, the stimulating and recording electrodes were inserted and the recording electrode was adjusted until a constant wave form was obtained. The rat was then asphyxiated by clamping the trachea. By holding the recording electrode in one position it was possible to trace the alteration of the action potential during advanced asphyxia and circulatory collapse.

RESULTS.

Fig. 1 shows the histogram for the duration of the muscle action potentials of conscious animals. The histogram in Fig. 2 shows the distribution of the duration from the control stimulated series. There is no statistical difference between the results obtained by the two techniques.

For 72 to 96 hours after adrenalectomy the duration of the action potential remained within normal limits (Fig. 3a). Six days after operation the peak of the histogram had shifted to 4–5 milliseconds (Fig. 3b). By seven days the animals usually showed clinical evidence of adrenal deficiency, being apathetic, sluggish, drinking little and frequently with the cold cyanosed extremities of peripheral vascular failure. The scatter around the mean duration had now increased, and the maximum duration was between five and six milliseconds (Fig. 3c). The readings after six days are classed statistically as significantly prolonged (1 p.c. significance). Those obtained after seven days are highly significant.

After adrenalectomy the rats rarely survived more than eight to ten days. This survival period was less variable than that reported by Grollman (1936b) and other workers (Frior and Grollman, 1933; Jaffe, 1926; Pencharz *et al.*, 1931), who report occasional survival for up to three months. The early death in our animals is thought to be due to the more complete removal accomplished

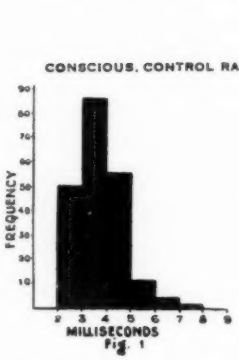

Fig. 1

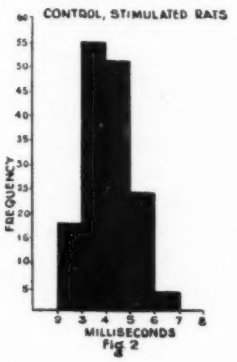

Fig. 2

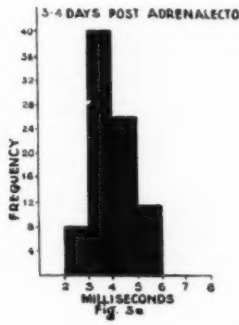

Fig. 3a

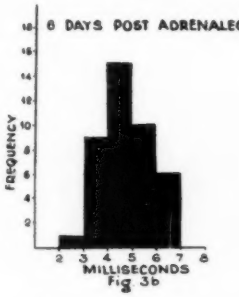

Fig. 3b

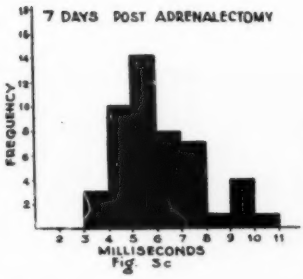

Fig. 3c

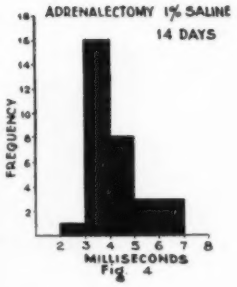

Fig. 4

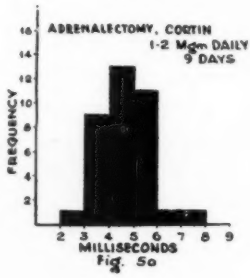

Fig. 5a

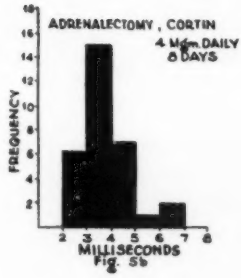

Fig. 5b

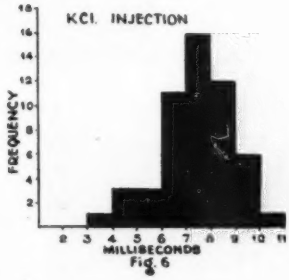

Fig. 6

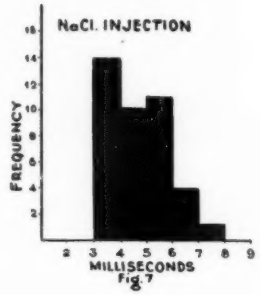

Fig. 7

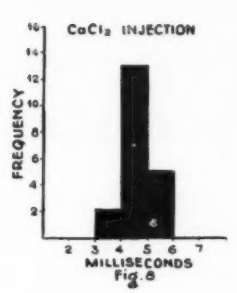

Fig. 8

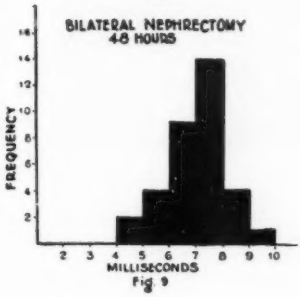

Fig. 9

by the anterior route, and the warm summer weather at the time the experiments were done. Britton (1931) showed the influence of the seasons on survival after adrenalectomy.

The effects of adrenalectomy can be prevented for as long as 14 days by substituting 1 p.c. saline (NaCl) for the drinking water (Fig. 4).

Small doses of cortical extracts (1–2 mg. desoxycorticosterone acetate, "Cortin," daily) prevented the weight loss and general deterioration of the animal, yet after nine days the electromyographic changes were similar to those occurring in six days in the untreated adrenalectomized animals (Fig. 5a). Larger doses (4 gm. daily) prevented any change of the action potential (Fig. 5b).

Potassium chloride injection produced gross increase of the duration of the action potential (Fig. 6). But equivalent doses of sodium or calcium chloride had little effect (Figs. 7, 8).

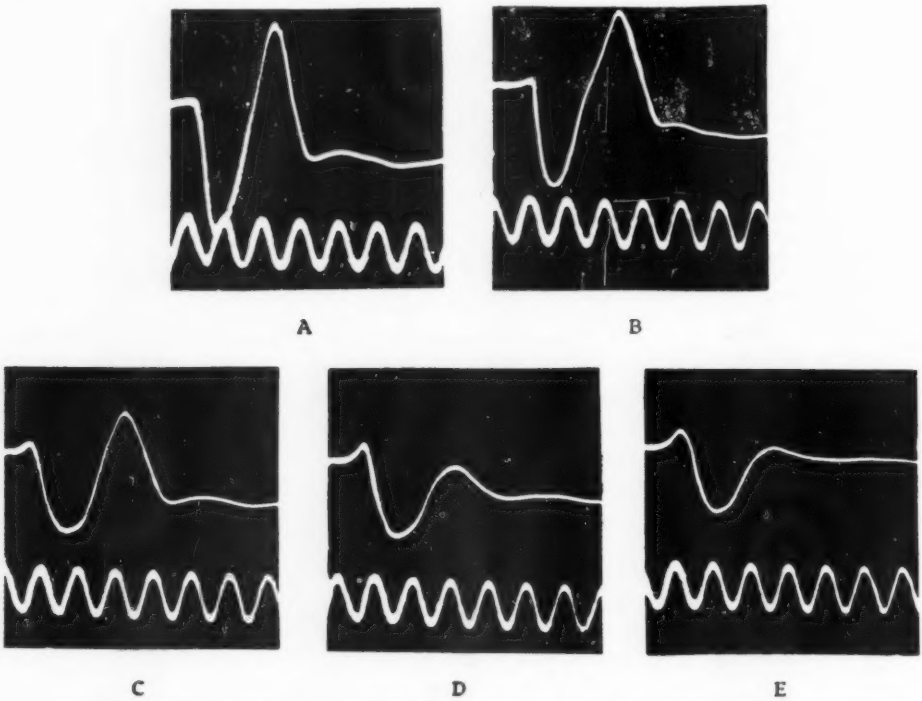

Fig. 10. Changes occurring in a muscle action potential with increasing acute and generalized anoxia. A. Normal. B. 1–2 min. after clamping the trachea. C. Respiratory efforts becoming weak, 1–2 min. later. D. Respiratory efforts and pulse ceased 1–2 min. later. E. 1 min. later, very weak muscle contractions.

Bilateral nephrectomy caused a marked increase of the duration similar to that due to potassium chloride (Fig. 9).

Fig. 10 shows the changes that occurred in an action potential as acute asphyxia became severe. The amplitude slowly decreased but even a few seconds before all activity ceased the duration of the action potential was little altered.

DISCUSSION.

The results of adrenal failure have frequently been, partly or entirely, ascribed to potassium poisoning (Alliers *et al.*, 1936; Hastings and Compere, 1931; Wilder *et al.*, 1937; Zenemer and Truszkowski, 1937). Harrison and Darrow (1938) showed that in rats after adrenalectomy the serum potassium increased to 7·4 milliequivalents per litre when the animal had clinical evidence of severe adrenal deficiency.

The animals injected with potassium chloride soon showed the clinical effects of potassium poisoning, and in the later stages there was marked peripheral circulatory failure. The prolongation of the muscle action potential was specific to potassium, because excess chloride and water produced by sodium or calcium chloride injections did not have the same effect. Calcium chloride in these doses also produced marked peripheral vascular collapse. The anoxia produced by strangulation did not prolong the muscle action potential. The potassium effect is therefore not secondary to muscle anoxia.

Bilateral nephrectomy produces a greater retention of potassium than does adrenalectomy (Coates and Wellby, 1952; Harrison and Darrow, 1938), and also produced a greater prolongation of the muscle action potential.

Anderson and her co-workers (1940) showed that the potassium excretion of adrenalectomized rats was restored to normal if the animals were given 1 p.c. saline as drinking water; this also prevented the alteration of the myograph.

Adrenocortical extracts are known to prevent the electrolyte changes following adrenalectomy (Ingle *et al.*, 1937; Thorn, 1939; Thorn *et al.*, 1936). Cortin also maintains the normal muscle power in adrenalectomized and nephrectomized rats, by decreasing the potassium accumulation, as it has no effect on the retention of urea, sodium or chloride (Ingle *et al.*, 1937). In the present study cortical extracts maintained the duration of the muscle action potential within normal limits.

Nastuk and Hodgkin (1950) in experiments *in vitro* showed that a decrease of the sodium content in the external medium caused a decreased amplitude, and their figures showed a prolongation of the muscle action potential. The decreased serum sodium was not an important factor in the present studies because nephrectomy which produces a sodium retention (Coates and Wellby, 1952) was followed by a marked increased duration of the action potential.

The important influence of potassium on the membrane potential of both nerve and muscle has been known for many years (Bernstein and Hober, quoted by Hodgkin, 1951; Ling and Gerard, 1949). Katz (1949) confirmed Overton's

observation (1904) that the muscle action potentials are eliminated by increasing the extra-cellular potassium. Potassium excess in experiments *in vivo* may cause a prolongation of the action potential by depressing the membrane potential, by altering the ionic balance on the two sides of the cell membrane, or by acting as a specific metabolic poison. The exact mechanism involved is not clear.

SUMMARY.

A method of obtaining electromyographs from rats is described.

The duration of the muscle action potential in the conscious and in the anaesthetized rat under the experimental conditions described was found to be the same.

Bilateral adrenalectomy was followed by an increased duration of the muscle action potential. This has been shown to be due to an accumulation of potassium in the body. The same result was produced by the injection of potassium chloride, but not by sodium or calcium chloride.

Bilateral nephrectomy also prolonged the muscle action potential.

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THE ISOLATION OF *COXIELLA BURNETI* FROM THE TICK *IXODES HOLOCYCLUS* IN QUEENSLAND

by J. G. CARLEY AND J. H. POPE

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(Accepted for publication 9th June, 1953.)

This laboratory has been interested in the recovery of infective agents from ectoparasites found naturally in Queensland. As they come to hand, ticks from widely separated localities have been inoculated into mice. Recently *Coxiella burneti* has been isolated from ticks on two such occasions.

The ticks, after identification, were suspended as completely as possible by trituration with normal saline containing a final concentration of 500 units of penicillin per ml. After allowing the larger particles to settle in the sterile mortar, 0·5 ml. of supernate was inoculated intraperitoneally into mice three to four weeks old.

One isolation was from eight female *Ixodes holocyclus* Neumann which were removed from his cows in August, 1952, by a dairy farmer at Julatten, North Queensland, who himself had had Q fever in December, 1951. The other was from three unattached nymphal *I. holocyclus* collected at Mt. Glorious (about 20 miles north-west of Brisbane) in September, 1952.

One of the four mice inoculated from the Julatten ticks showed an enlarged spleen, and splenomegaly was seen in a large proportion of mice in subsequent passages. Neither of the two mice originally inoculated from the Mt. Glorious ticks had an enlarged spleen when examined at 14 days, but splenomegaly was regularly seen in passage mice. Rickettsiae, in the colony formation characteristic of *C. burneti,* were demonstrated in Giemsa-stained spleen smears of mice infected with each strain.

After nine passages in mice, the strains were studied through several passages in guinea-pigs. These usually showed a mild febrile reaction after an incubation period of 4 to 15 days. The sera from two guinea-pigs from each strain were tested at the Laboratory of Microbiology and Pathology for agglutinating and complement-fixing antibodies, with results as shown in Table 1.

Seven months after the isolation of the Julatten strain, a re-test of the same batch of ticks was made. Nineteen ticks that had been stored at —15° C. were injected into two guinea-pigs, but the results were negative.

Results with ticks examined in this Institute for infective agents by inoculation into mice and guinea-pigs are shown in Table 2. All examinations were negative except for the two batches from which *C. burneti* was obtained.

TABLE 1.

Antibody response to strains of Coxiella burneti *in sera of guinea-pigs.*

Strain	Guinea-pig No.	Days after inoculation	Reciprocal titre of antibodies	
			Agglutinating	Complement-fixing
Julatten	974	22	40	8
	975	32	80	16
Mt. Glorious	970	22	20	<8
	971	32	80	64

TABLE 2.

Ticks examined for infective agents.

Species	No. of ticks inoculated	No. of batches
Ixodes holocyclus Neumann	159	27
Ixodes tasmani Neumann	221	8
Ixodes fecialis Warburton and Nuttall	1	1
Boophilus microplus Canestrini	84	2
Amblyomma moreliae (L. Koch)	1	1
Amblyomma triguttatum Koch	51	1
Aponomma hydrosauri (Denny)	9	1
Ornithodoros gurneyi Warburton	30	1
Rhipicephalus sanguineus (Latreille)	5	1

DISCUSSION.

This report adds one more to the number of tick species in the world—at least 17 (Derrick, 1953)—which have been found naturally infected with *C. burneti.*

I. holocyclus is the tick which most commonly attacks man in Queensland. Smith (1942) demonstrated that it could be experimentally infected with *C. burneti* at all stages, and could transmit Q fever in the laboratory. Natural infection was not found among the 220 ticks Smith removed from captured bandicoots.

Acknowledgments. We are greatly indebted to Dr. F. H. S. Roberts for the identification of the ticks, to Mr. H. E. Brown and Mr. D. J. W. Smith for the serological tests, to Miss P. E. Lee for assistance with the mouse studies, and to Dr. E. H. Derrick for advice.

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THE ACTION OF UREA ON THE URINARY INHIBITOR OF INFLUENZA VIRUS HAEMAGGLUTINATION

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(Accepted for publication 9th June, 1953.)

The action on proteins of concentrated urea solutions has been widely studied. Three main types of change have been shown to occur; dissociation of the protein into more or less definite sub-units, aggregation and the unfolding of the globular proteins (see review by Neurath *et al.*, 1944). Usually these changes are accompanied by a loss of biological activity.

Burnet (1952) has found that treatment with strong urea solutions of a mucoprotein inhibitor of influenza virus haemagglutination isolated from human urine (urinary inhibitor) left its inhibitory function and immunological properties unchanged, but destroyed the inhibitor's haemagglutinin. The action of urea also markedly increased the solubility of the inhibitor in salt solutions.

This paper will present some details of the preparation of urea treated urinary inhibitor (U.U.I.) and the results of studies on its viscosity and solubility.

Materials and Methods.

Buffers and salt solutions. Analytical reagent grade salts were used throughout. The buffers were as given in an earlier paper (Curtain, 1953a), with the inclusion of the following:

pH 2, 0·25M KH phthalate, 0·25M HCl.
pH 1, 0·1 N HCl.

Lee virus and the receptor destroying enzyme of Vibrio cholerae *(RDE)* were prepared by the method of Gottschalk and Perry (1951) and Ada and French (1950) respectively.

Urinary inhibitor (U.I.) was prepared and treated with Lee virus or RDE as described previously (Curtain, 1953a).

Haemagglutination inhibitor titrations were carried out by the method of Stone (1951).

Titration of U.I. haemagglutinin was carried out using either RDE treated human or fowl erythrocytes according to the method given by Burnet (1952).

Viscosity, partial specific volume and solubility measurements were carried out using the apparatus and methods described previously (Curtain, 1953a).

EXPERIMENTAL.

Conditions affecting the preparation of U.U.I.

Concentration of urea and duration of treatment. Solutions of U.I. (0·5 p.c. w/v) were made up to different urea concentrations and held at 0° C. for varying periods. They were then placed in "Visking" sausage casings and

[1] This work was aided by a grant from the National Health and Medical Research Council, Canberra.

TABLE 1.

Effects of treatment with urea for varying concentrations and times on the haemagglutinin (HA) and inhibitory (I) titres and the axial ratio (a/b) of U.I. (conc. 0·2 p.c. w/v).

Molarity of urea	4 hours			8 hours			16 hours			24 hours		
	HA×10³	I×10³	(a/b)	HA×10³	I×10³	(a/b)	HA×10³	I×10³	(a/b)	HA×10³	I×10³	(a/b)
Normal U.I. control	300	20	150	300	20	150	300	20	150	300	20	150
4·5 M	300	20	150	256	20	150	100	20	150	75	20	130
5·0 M	300	20	150	200	20	150	100	20	130	50	20	127
5·5 M	300	20	150	200	20	150	75	20	130	40	20	127
6·0 M	300	20	150	128	20	135	64	20	120	20	20	75
6·5 M	300	20	150	150	20	140	3	20	50	±0·1	15	20
7·0 M	100	15	100	20	4	50	±0·1	0·5	10	<0·1	<0·1	10

dialysed in the cold for 48 hours. The inhibitory and haemagglutinin titres, dry weight, partial specific volume and viscosity of each of the dialysed solutions were determined. The partial specific volume of U.U.I. was found to be the same as that of U.I. (0·705). The viscosity increment (ν) was calculated from the Einstein equation,

$$\nu = \frac{1}{\phi}\left(\frac{\eta}{\eta_0} - 1\right)$$

where ϕ is the volume fraction of the solution occupied by the U.U.I. and $\frac{\eta}{\eta_0} - 1$ the specific viscosity of the solution.

The value for ν was substituted in the Simha (1939) equation to give the axial ratio (a/b), assuming the molecule of U.I. to be an unhydrated prolate ellipsoid (see discussion, Curtain, 1953a).

$$\nu = \frac{(a/b)^2}{15\,(\log 2(a/b) - 3/2)} + \frac{(a/b)^2}{5\,(\log 2(a/b) - 1/2)} + 14/15$$

The axial ratio was compared with the inhibitory and haemagglutinin titres for each urea concentration and length of treatment (Table 1).

It can be seen that there is a progressive drop in axial ratio with increasing concentration of urea. The inhibitory titre is unaffected until a urea concentration of 6·5 M is reached, above which it drops markedly, being completely destroyed by the action of 7 M urea for 24 hours. Rapid destruction of the haemagglutinin occurs at urea concentrations above 6·5 M provided the solutions are treated for 16 hours or more.

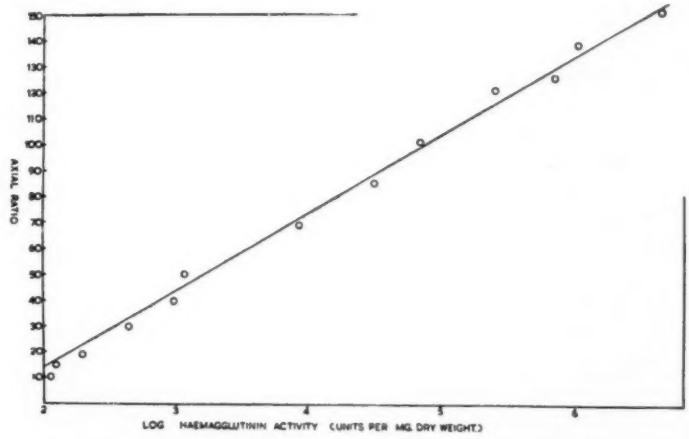

Fig. 1. Relation between the axial ratio and the logarithm of the haemagglutinin of urea treated U.I.

The results indicated that there might be some direct relation between the axial ratio and the haemagglutinin titre, and an experiment was designed to test this hypothesis. Solutions of U.I. were treated with varying concentrations of urea for 16 hours and dialysed against several changes of distilled water for 36 hours with stirring. The haemagglutinin titres (in duplicate), viscosities and dry weights of the solutions were determined. The logarithm of haemagglutinin activity (units per mg. dry weight of the urea treated inhibitor) is plotted against the axial ratio in Fig. 1. The regression equation was calculated and its significance tested according to the methods given by Fisher (1948). The straight line obtained indicated that under the conditions of the experiment there was a linear relation between the axial ratio and the logarithm of the haemagglutinin.

Generally, the results obtained indicate that U.U.I. with very low haemagglutinin and minimum destruction of inhibitory titre is only produced over a narrow range of urea concentration.

Effect of pH and ionic strength. In Fig. 2 are shown the effects of pH and ionic strength on the reduction of viscosity by treatment with 6·5 M urea for 24 hours. The action of urea is reduced by increasing the ionic strength and reducing the pH. It was found that at ionic strengths above 0·20 the viscosity and haemagglutinin titre were unaffected by any urea concentration acting for periods of up to 72 hours.

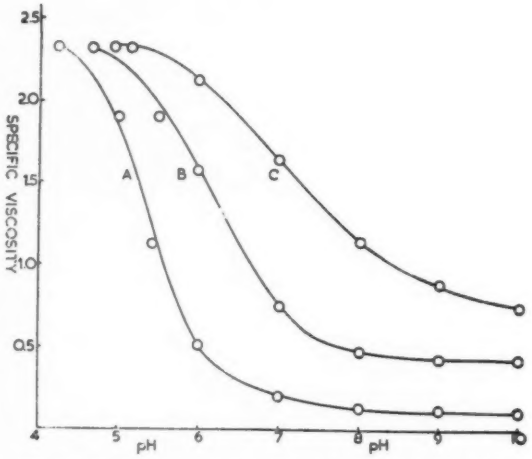

Fig. 2. Reduction of the viscosity of U.I. by 6·5 M urea acting for 24 hours.

Effect of pH and ionic strength.
Curve A, ionic strength, μ, = 0·05
Curve B, μ = 0·10
Curve C, μ = 0·15

Effect of alkaline earth cations. Fig. 3 shows the effect of 6·5 M urea on the viscosity of U.I. in the presence of varying concentrations of $CaCl_2$, $MgCl_2$, and $BaCl_2$ (time of treatment, 24 hours). The results show that increasing concentrations of these salts cause a progressively greater decrease in the reduction of viscosity. Higher concentrations (Ca, ionic strength, μ, = 0·023; Mg, μ, = 0·017; Ba, μ, = 0·013) completely inhibit the action of urea on the viscosity, haemagglutinin and inhibitory activity.

Effect of prior incubation of U.I. It was found that the stability of the inhibitor activity to urea was influenced by the past history of the U.I. Solutions which had been aged by incubation at 37° C. under sterile conditions for several hours or by standing for a longer time at lower temperatures lost their inhibitory titre on treatment with urea at concentrations as low as 5 M and for times as short as 10 hours.

Standard method of preparing U.U.I.

The above results indicated that specific conditions with respect to urea concentration, time of treatment and concentration and nature of electrolyte were necessary to produce U.U.I. which had the lowest haemagglutinin consistent with the minimum destruction of inhibitory titre. For the remaining experiments U.U.I. was prepared by treating an 0·3–0·5 p.c. (w/v) solution of freshly prepared U.I. in distilled water with 6·5 M urea for 24 hours at 0° C. followed by dialysis with stirring against several changes of distilled water for 48 hours. These preparations had haemagglutinin titres of <100 and minimal loss of inhibitory activity.

Viscosity and solubility of U.U.I.

Effect of pH and ionic strength. Fig. 4 shows the effect of pH on the viscosity of U.U.I. It can be seen that a break in the curve occurs between pH 3·3 and 2·8. This corresponds to the pH range of minimum solubility (0·02 p.c. w/v). The solubility of U.U.I. is independent of the pH outside of the range 3·3–2·8. Solutions of up to 10 p.c. (w/v) were obtained by dissolving freeze dried material in a suitable volume of buffer.

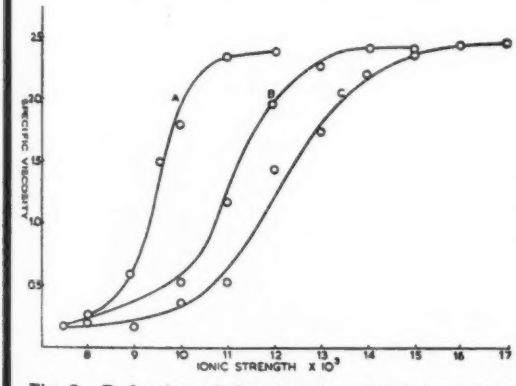

Fig. 3. Reduction of the viscosity of U.I. by 6·5 M urea acting for 24 hours.
Effect of varying concentrations of the alkaline earth cations.
Curve A, $BaCl_2$. Curve B, $MgCl_2$. Curve C, $CaCl_2$.

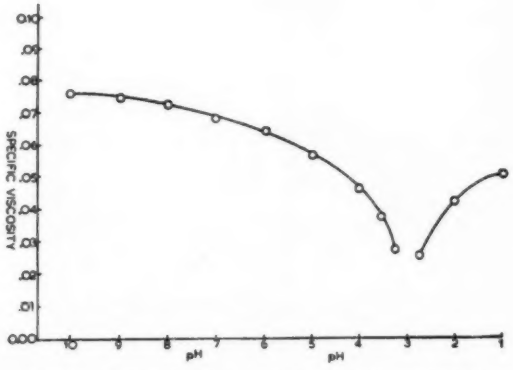

Fig. 4. The dependence of the viscosity of U.U.I. on pH. The break in the curve represents the pH range of minimum solubility of U.U.I.

Increasing ionic strengths had little effect on the viscosity or solubility. At ionic strengths greater than one a slight decrease was observed in the viscosity, and with the aid of ammonium sulphate it was possible to salt out U.U.I. at ionic strengths greater than 6.

Effect of the alkaline earth cations. In contrast to their action on normal U.I. (Curtain, 1953a) the alkaline earth cations, calcium, magnesium and barium, have no effect on the viscosity and solubility of U.U.I.

Effect of treatment with RDE or Lee virus. U.U.I. was treated with RDE and Lee virus under the same conditions as used for U.I. (see Curtain, 1953a). The variation of solubility and viscosity with ionic strength was the same as for untreated U.U.I., but the pH of minimum viscosity and solubility was increased to approximately 4·5 after RDE treatment, and 4·3 after Lee virus treatment.

In keeping with the finding of Burnet (1952) with normal U.I., the action of urea on RDE or Lee treated U.I. did not increase the inhibitory titre. It was also found that the order of treatment of U.I. with urea or RDE had no effect on the viscosity and solubility of the final product.

Effect of incubation at 37° C. In the experiments with U.I. it was observed that incubation of the solutions at 37° C. for varying periods in the presence of salts led to an increase in viscosity. This effect was also noticed with U.U.I. and was accompanied by an increase in the haemagglutinin titre. In Table 2 are given the results of some experiments in which U.U.I. was heated for various times at 37° C.

TABLE 2.

Incubation of U.U.I. and U.I. control (concentrations 0·2 p.c. w/v) for varying periods in 0.05 N KCl at 37° C.

Time, hours	Haemagglutinin	Inhibitor $\times 10^3$	η/η_0-1
0	10	16	0·06
1	50	16	0·06
2	50	16	0·06
4	300	16	0·07
8	900	16	0·09
16	1,500	16	0·10
24	3,200	16	0·10
Normal U.I.			
0	300,000	32	2·2
24	300,000	32	3·3

It can be seen that incubation only slightly increases the viscosity of U.U.I. and that the haemagglutinin titre is only increased to 1 p.c. of its former value.

The action of guanidine and thiocyanate on U.I.

Since strong solutions of guanidine hydrochloride and ammonium thiocyanate have been reported to have the same depolymerizing effect on asymmetric proteins as urea (e.g. myosin, Edsall and Mehl, 1940) the effect of these

reagents on U.I. was investigated. It was found that even saturated solutions of guanidine hydrochloride or ammonium thiocyanate had no effect on the haemagglutinin and inhibitory titres or the viscosity.

DISCUSSION.

Burnet (1952) has discussed the nature of the U.I. agglutinin and its reduction following treatment with urea, particularly in the light of the increased agglutination by normal U.I. of red cells after RDE treatment. Such cells have a decreased net negative charge suggesting that the increased agglutinability may be due to the enhanced binding of the negatively charged U.I. molecules. Indeed, the negatively charged colloidal dye Congo red showed the same behaviour towards normal and RDE treated cells as normal U.I. whilst positively charged protamine agglutinated both to the same titre. However, destruction of the agglutinin by urea is not accompanied by a change in the net negative charge of the molecules as determined by electrophoresis (Pye, 1954). Thus, other factors beside charge must play a part in U.I. haemagglutination. The present work shows that the logarithm of the haemagglutinin titre is linearly related to the axial ratio of the urea treated inhibitor. U.I. almost completely loses its haemagglutinin when its axial ratio is reduced to a level which corresponds to a molecular weight of approximately 5×10^5. The molecular weight of the Congo red micelle has been stated to be 8×10^3 (Fieser and Fieser, 1944). It appears, therefore, that the size, shape and charge of the agglutinin may all play a part in haemagglutination.

The marked increase in the viscosity of U.I. after treatment with urea is comparable with that observed in the case of tobacco mosaic virus (Frampton and Saum, 1939) and myosin (Edsall and Mehl, 1940) where breakdown of asymmetric particles occurs to give solutions of lowered viscosity. A comparison of the viscosity and solubility of U.I. and U.U.I. shows that while the latter behaves like a typical protein the former shows several anomalies (Curtain, 1953a). Thus, the viscosity of U.U.I. approaches a minimum near the isoelectric point and decreases with increasing ionic strength but is unaffected by treatment with virus or RDE (except for the differences due to change in isoelectric point) or by the presence of the alkaline earth cations. The solubility of U.U.I. is unaffected by ionic strength, alkaline earth metals and pH, except over the narrow pH range of minimum solubility. The action of RDE or virus merely changes the position of this range. These changes in the behaviour of U.I. after treatment with urea are probably due to the reduction of the axial ratio and consequent diminution of the London–van der Waals forces of molecular interaction (see Discussion, Curtain, 1953a).

The loss of ionic strength and pH sensitivity makes U.U.I. a more suitable inhibitor than U.I. for many studies, such as electrophoresis and acid base titrations. Both of these techniques are being applied to the elucidation of the

nature of the reduction in net negative charge caused by the action of RDE or virus on their substrates. The results of acid base titrations carried out on U.I. and U.U.I. will be discussed in a subsequent paper (Curtain, 1953b).

SUMMARY.

A study has been made of the action of urea on the physical and biological properties of a mucoprotein inhibitor of influenza virus haemagglutination isolated from human urine (U.I.). It was found that increasing concentrations of urea progressively diminished the axial ratio of U.I. (as determined by viscosity measurements) and its haemagglutinin titre. At urea concentrations above 6·5 M the inhibitory titre was also reduced. In contrast to normal U.I., urea treated U.I. is soluble in strong salt solutions and solutions of the alkaline earth metal salts and its viscosity and solubility are independent of pH, except over a narrow range about the isoelectric point. Because of these properties urea treated U.I. is considered to be more suitable than normal U.I. for many studies where solubility over a wide range of pH and ionic strength is desirable.

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THE NATURE OF THE BOND SPLIT BY THE INFLUENZA VIRUS ENZYME

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Hanig (1948) showed by microelectrophoresis that the action of PR8 influenza virus caused a marked reduction in the net negative charge of the human erythrocyte. It was later found (Ada and Stone, 1950a, 1950b; Stone and Ada, 1950) that this reduction in negative charge was brought about to a varying extent by the other strains of influenza virus and the receptor destroying enzyme of *Vibrio cholerae* (RDE).

These observations were extended by Perlmann, Tamm and Horsfall (1952) and Pye (1954) to the soluble inhibitors of influenza virus haemagglutination. With every inhibitor studied it has been shown by electrophoresis that the action of RDE or virus causes a reduction in the net negative charge of the molecule. Since all the evidence (see review by Burnet, 1951) supports the hypothesis that the reduction in charge of both the red cell and the inhibitors is due to the virus enzyme, it appeared likely that a more detailed study of the change in charge might yield information on the nature of this enzyme and its action. Electrophoresis can only measure the diminution of the net negative charge, it cannot indicate the number and nature of the groups involved. This information can be obtained, however, by carrying out acid-base titrations on a suitable virus receptor carrying substrate before and after the action of virus or RDE.

Early attempts in this direction were unsuccessful because of the lack of a pure substrate. Due to the very large reduction in the electrophoretic mobility of red cells after RDE action (from 1·30 to 0·17 μ/sec./volt/cm.), it was considered that acid-base titration of stromata might give some information.

Although the virus receptors are in the mobility determining (shear) layer of the red cell membrane, it was found that they represented only a small fraction of the titratable constituents of the stromata (including the non-mobility determining groups in layers other than the shear boundary, Curtain, 1951). The inhibitor isolated by Tamm and Horsfall (1950, 1952) from human urine provided the first readily available homogenous substrate for RDE and the influenza virus enzymes. In its original form this inhibitor was not completely suitable for acid-base titration since it was insoluble at pH values below 4·8, making it impossible to obtain the acid branch of the titration curve. It was found, however, that treatment of the inhibitor with urea (Burnet, 1952) gave a product which had full inhibitory activity and was insoluble over only

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a narrow range of pH (pH 2·9–3·1 for normal urea treated inhibitor and pH 3·8–4·0 after treatment with RDE, Curtain, 1953b). Pye (1954) has shown that the action of RDE causes a reduction in net negative charge of this urea treated inhibitor.

This paper presents the results of acid-base titrations of normal and urea treated urinary inhibitor before and after the action of RDE and an interpretation in terms of the number and nature of the ionising groups affected.

Materials.

Normal hydrochloric acid was prepared by diluting A.R. concentrated hydrochloric acid with distilled water and standardizing this solution against three times recrystallized sodium metaborate.

Normal potassium hydroxide was prepared by standardizing against twice recrystallized potassium hydrogen phthalate, a solution made from A.R. potassium hydroxide pellets and distilled water.

The normality of these acid and alkali solutions was checked by titration against each other.

Potassium hydrogen phthalate. An 0·05 molar solution was prepared from distilled water and A.R. potassium hydrogen phthalate which had been twice recrystallized from distilled water and dried in air. The pH of this solution is equal to 4·005 at 25° C. (Hamer, Pinching and Acree, 1946).

Sodium metaborate. An 0·01 molar solution was prepared from distilled water and A.R. sodium metaborate, which had been recrystallized from distilled water three times and dried in air. The pH of this solution is equal to 9·177 at 25° C. (Manov, DeLollis, Lindvall and Acree, 1946).

0·1 Normal hydrochloric acid. This was prepared by diluting the normal HCl in distilled water. The pH is equal to 1·085 at 25° C. (Hitchcock and Taylor, 1938).

Urinary inhibitor (U.I.) was prepared by a previously described (Curtain, 1953a) modification of the method of Ada and Gottschalk (1952).

Urea treated urinary inhibitor (U.U.I.) was prepared from this following the method given previously (Curtain, 1953b).

Inhibitor treated with RDE or Lee virus. The U.I. and U.U.I. were incubated with a purified preparation of RDE (Ada and French, 1950) under conditions given previously (Curtain, 1953a and b). U.I. treated with Lee virus was also prepared (Curtain, 1953a).

Preparation of inhibitor solutions for titration. Inhibitor solutions were dialyzed exhaustively against 0·05 ionic strength KCl in the case of U.I. and 0·1 ionic strength KCl in the case of U.U.I. U.I. solutions were titrated with 0·05 N acid or alkali and U.U.I. with 0·1 N acid or alkali, thus maintaining the total ionic strengths at 0·05 and 0·1 respectively. Dry weights were determined by drying in an air oven at 110° C., correction being made for salt present in original solution.

Apparatus.

Titrations were carried out using the glass electrode assembly described by Holden (1938).

The potentiometer was constructed by Mr. Holden from a modification of the Harrison (1930) circuit. The changes include the use of a Cambridge moving spot galvanometer which also serves for the standardization of the potentiometer, a variable galvanometer shunt instead of a shunting switch and a Muirhead type D-2 four dial voltage dividing resistance. The instrument is capable of reading accurately to 0·1 millivolt, this being sufficient to give a precision of 0·005 pH unit with a temperature controlled cell.

The titration cell was maintained at 25 ± 0·05° C. in a lagged bath through which kerosene is pumped from a remote thermostatically controlled container. Kerosene was chosen as the thermostat liquid since the use of water would lead to an undesirable increase in humidity in the neighbourhood of the glass electrode and potentiometer. The bulk container with the heater and pump was placed at least three feet from the cell and potentiometer in order to isolate inductive fields due to the heater and pump motor circuits.

Glass electrodes were blown from Corning 015 glass to a bulb resistance of not more than 25 megohm. The electrodes were sealed with polythene and then coated with silane as recommended by Gilbert (1952). Silver/silver sulphate internal reference electrodes were used.

Since glass electrodes made from Corning 015 glass are unsatisfactory at pH values above 9, the hydrogen electrode was used to calibrate these electrodes for the alkaline range of the titration. Cylinder hydrogen was used after purification by bubbling through alkaline potassium permanganate, 20 p.c. sodium hydroxide and concentrated sulphuric acid. Oxygen was removed by passage over a hot tungsten wire. A Hildebrand type of platinized electrode was placed directly in the titration cup.

EXPERIMENTAL.

Titration technique. Titrations were carried out by adding aliquots of the hydrochloric acid or potassium hydroxide to the inhibitor solution in the titration cup. The mixture was stirred by means of a small electric stirrer, the liquid junction opened and the potential of the cell determined.

Standardization of the pH scale. The equation relating pH to the electromotive force, E, developed by the glass electrode cell is given by the relation $pH = -\log_{10} a_{H^\bullet} = \frac{E - E'}{2{\cdot}303\,RT/F}$ where E′ is the sum of all potentials in the cell and RT and F have their usual significance. In order to standardize the pH scale with respect to any given cell, it is necessary to evaluate E′ by the use of solutions of known pH. The borate, phthalate and 0·1 N hydrochloric acid were used for this purpose. At 25° C. identical values of E′ were obtained with all three solutions. During titrations the value of E′ was checked by frequent reference to these standards.

Method of calculation.

The number of equivalents of hydrogen ion bound at any pH by the inhibitor was obtained from the difference between the number of equivalents of $H^\bullet$ or OH^- added and the number of equivalents of free $H^\bullet$ or OH^- present at that pH. The values for free $H^\bullet$ and OH^- were computed on the assumption that:

$$pH = -\log_{10} (H^\bullet)_{f'H^\bullet} = \frac{-\log_{10} (OH^-) \times f'_{OH^-}}{K_W}$$

where the value of K_W ($1{\cdot}008 \times 10^{-14}$) given by Harned and Owen (1939) was used. The activity coefficients have been designated *f′* because they are different from activity coefficients determined by any other method. To obtain the molarity of the free $H^\bullet$ and OH^- in the inhibitor solutions it was assumed that the activity coefficients depended only on the inorganic ions in the solution. The activity coefficients in the absence of inhibitor were determined by measuring

the pH of solutions of known concentrations of HCl and KOH in the presence of KCl at the ionic strength used in the titrations.

Expression of results. All results are expressed in terms of milli-equivalents of H^+ dissociated (or "H^+ bound and OH^- bound") per gm. dry weight of inhibitor.

Reversibility of the titration.

Four criteria of reversibility were applied.

1. The stability of the potential of inhibitor solutions held at various pH values was observed for periods of not more than thirty minutes at 25° C. Previous experience has shown that the inhibitory titre of both U.I. and U.U.I. rapidly decreases during exposure to pH values above 10 for periods of longer than one hour.

2. The solutions were back titrated from both extremes of the titration curve. This was of particular value in determining the validity of the curves beyond the pH ranges of insolubility of normal and RDE treated U.U.I. The titrations were regarded as reversible if the forward and back titrations agreed to within 0·05 pH unit.

3. In the case of U.I. the electrophoretic mobility of the normal and the RDE and virus treated inhibitors was determined after back titration to neutrality.

4. The back titrated U.I. and U.U.I. were examined for any decrease in inhibitory activity.

The titration of U.I. before and after treatment with RDE or Lee virus.

The results of the titrations are shown in Fig. 1.

The titration was not carried out below pH 6 owing to the insolubility of RDE treated U.I. The three curves were reversible throughout the entire alkaline range by all the criteria given in the previous section. It can be seen that there is an increase in the maximum hydrogen ion dissociation ("base binding capacity") of the inhibitor after virus or RDE treatment. The RDE and virus curves are almost identical; both begin to show

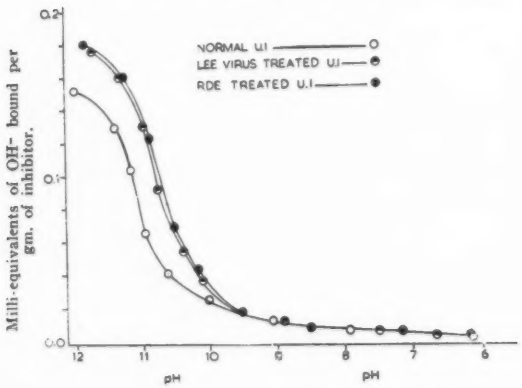

Fig. 1. Titration curves of U.I. before and after treatment with RDE or Lee virus.

significantly increased hydrogen ion dissociation as compared with normal U.I. at approximately pH 10. In contrast to the titrations of many other proteins the three curves appear to tend to a true maximum in their hydrogen ion dissociation (at about pH 11·8).

The titration of normal and RDE treated U.U.I.

These results are given in Fig. 2. It can be seen that the alkaline branches of the curve are similar to those obtained with normal and RDE treated U.I.

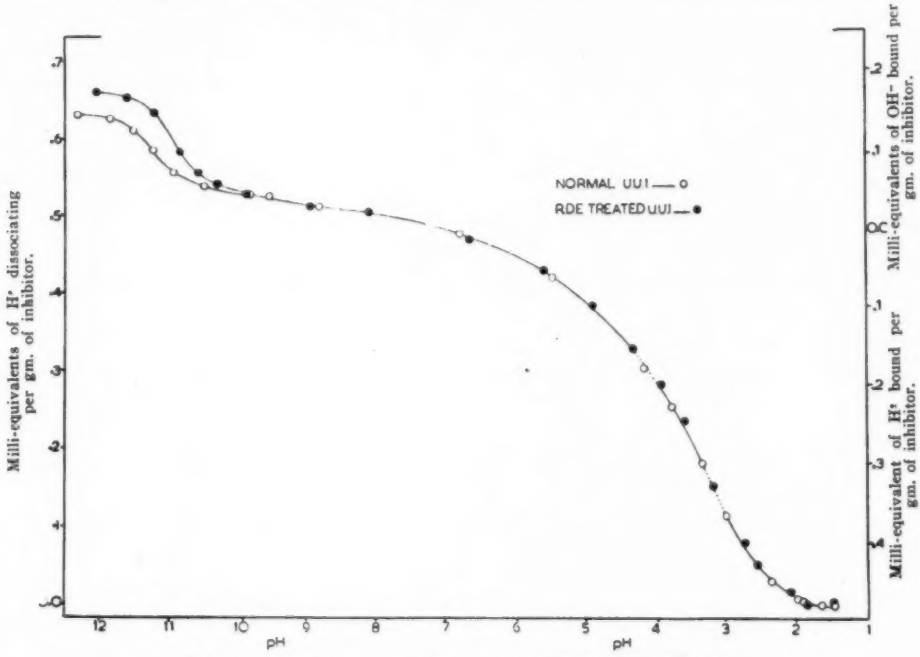
NORMAL UUI
RDE TREATED UUI
Milli-equivalents of H' dissociating per gm. of inhibitor.
Milli-equivalent of H' bound per gm. of inhibitor.
Milli-equivalents of OH- bound per gm. of inhibitor.
pH
pH

Fig. 2. Titration curves of normal and RDE treated U.U.I.

A complete acid titration curve was obtained save for two short breaks at pH 3·8–4·3 (due to the insolubility of RDE treated U.U.I.) and 3·0–3·3 (due to the insolubility of normal U.U.I. These breaks are shown by dotted lines on the curves. Except for these discontinuities the acid curves for the normal and RDE treated inhibitors are identical, both tending towards the same maximum hydrogen ion binding capacity below pH 2·3.

Both the acid and alkaline titrations were shown to be reversible by all the criteria given above, excepting electrophoresis of the back titrated material which was not carried out.

DISCUSSION.

The titration curve of U.U.I. is simple, showing only four points of inflection. This shape indicates the presence, in any quantity, of only two kinds of groups, those ionising in the acid range with a pK value of approximately 3·6 and those in the alkaline range with a pK of 11·2. These values were obtained by taking the mid point on the curve between the two inflections in each range. The value for the acidic groups agrees well with the figure for carboxyl groups whilst the pK of the basic groups, although a trifle high, falls within the range given for ϵ amino groups (see Cohn and Edsall, 1943). It is in the increased number of the latter groups that the RDE treated U.U.I. differs from the normal. This also applies to U.I. treated with either RDE or Lee virus, the alkaline curves of which are very similar to each other and to the RDE treated U.U.I. This and the similarity of the alkaline titrations of normal U.I. and U.U.I. indicate that RDE and the Lee virus enzymes have similar actions and also show that the action of urea causes no alteration in the number of amino groups dissociating in U.I. The small differences in shape between the alkaline curves of normal U.I. and U.U.I. are probably due to changes in the free energy of ionisation of the groups caused by reduction in the axial ratio of the molecule after urea treatment (Onsager, 1949). It appears, therefore, that the decrease in net negative charge found by electrophoresis of the inhibitors (and the erythrocyte) is due to the unmasking by RDE or the virus enzyme of positively charged ϵ amino groups. Since the separation of the curves does not occur until pH 10 no α amino groups can be involved since the pK value of these is not known to exceed 9. Since the only known amino acid with an ϵ amino group is lysine, it seems probable that the enzymic action on the inhibitors is concerned with the splitting of a bond involving the amino group of lysine. It is most likely that this is a peptide bond and that the receptor destroying and virus enzymes are specific peptidases.

Assuming the molecular weight of U.I. as 7×10^6 (Tamm and Horsfall, 1952) it is possible to calculate the number of groups changed per molecule by enzymic action. The difference in maximum hydrogen ion dissociation between the curves is 0·00004 equivalents of $H^{\cdot}$ per gm. of dry mucoprotein. Multiplying this by the molecular weight gives a figure of 280 equivalents per mole. This is a rough agreement with the figure of 200 substrate groups obtained by Gottschalk (1952) from chemical evidence.

Some experiments reported by Burnet (1952) lend further support of a qualitative nature to the contention that the receptor destroying and virus enzymes unmask amino groups on their substrates. Burnet found that the agglutination of red cells by U.I. and the colloidal dye Congo red was enhanced markedly by treating the cells with RDE. Both Congo red and U.I. are negatively charged at neutral pH values and it was thought that the agglutination

was due to the interaction of these with positively charged groups on the surface of the cell. This was supported by the fact that positively charged protamine which is a haemagglutinin for normal cells (presumably by interacting with the negatively charged groups of the cell surface) showed no increased activity towards RDE treated red cells. It was also shown that the agglutination by U.I. and Congo red could be abolished by adding formalin to the system. This agrees with the evidence presented in this paper that the action of the receptor destroying and virus enzymes on their substrate unmasks amino groups.

SUMMARY.

Acid-base titrations have been carried out on normal and urea treated urinary inhibitor of influenza virus haemagglutination (U.I. and U.U.I.) before and after the action of either the receptor destroying enzyme of *Vibrio cholerae* (RDE) or Lee virus. The titrations show an increase in the number of positively charged ionising groups in the inhibitor molecule after RDE or virus treatment, explaining the reduction in net negative charge which has been observed on electrophoresis of the treated inhibitors. It is suggested that the positively charged groups involved are the ϵ amino groups of lysine and that the receptor destroying and virus enzymes have a similar action in that they are specific peptidases.

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